

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Food ingredients from cultivated seaweeds  
Improving storage stability and protein recovery

HANNA HARRYSSON



Food and Nutrition Science  
Department of Biology and Biological Engineering  
CHALMERS UNIVERSITY OF TECHNOLOGY  
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# FOOD INGREDIENTS FROM CULTIVATED SEaweEDS

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Department of Biology and Biological Engineering

Food and Nutrition Science

Chalmers University of Technology

SE-412 96 Gothenburg

Sweden

Telephone: + 46 (0) 31 772 10 00

Cover: Cultivated *Porphyra umbilicalis*, *Ulva lactuca* and *Saccharina latissima* as sources for potential food ingredients. Illustrated by Kenth Harrysson.

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# FOOD INGREDIENTS FROM CULTIVATED SEaweEDS

## Improving storage stability and protein recovery

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### ABSTRACT

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There is a global demand for new vegetarian protein sources, and seaweed have for multiple reasons been identified as a promising candidate. The overall aim of this thesis was to evaluate the potential of three different Swedish seaweed species as food protein sources, but also as sources of unsaturated fatty acids, vitamins and minerals. To accomplish this, the goals were to i) obtain a seaweed biomass high in protein and lipids, ii) find strategies to maintain the quality of the seaweed biomass during drying and storage and iii) develop an efficient method for recovering seaweed proteins.

The protein and lipid content in seaweed is related, e.g. to the nutrient access and physical parameters of the surrounding environment. By applying alterative cultivation, the protein and fatty acid content in *U. lactuca* were increased 3.4 respective 1.5 times by nitrate addition. Furthermore, the protein content was increased by cultivation at low temperature (12 °C) and light (50  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ), and the fatty acid content was increased by low light and high temperature (18°C).

The polyunsaturated fatty acids (PUFAs) in seaweed could make the dried biomass susceptible to lipid oxidation, with co-oxidation of pigments and vitamins. However, during long-term storage ( $\leq 520$  days) of oven- and freeze-dried *P. umbilicalis* and *U. lactuca*, there was only a moderate development of the lipid oxidation-derived aldehydes, malondialdehyde, 4-hydroxy-trans-2-hexenal and 4-hydroxy-trans-2-nonenal, while there was a great loss of unsaturated fatty acids and ascorbic acid. Light stimulated the fatty acid loss as well as bleaching of chlorophyll.

Several advantages are foreseen from concentrating the seaweed proteins. This calls for food grade and scalable fractionation methods. The pH-shift process, using alkaline protein solubilisation followed by isoelectric precipitation, was in this work adapted and improved for *P. umbilicalis*, *U. lactuca* and *S. latissima*, e.g. by including freeze-thawing-stimulated protein precipitation. When then comparing the new pH-shift process to two other fractionation methods, the pH-shift method resulted in extracts with the highest protein content: 71%, 51% and 41% per dry weight for *P. umbilicalis*, *U. lactuca* and *S. latissima*, respectively. The protein contained 37-41% essential amino acids. The highest achieved protein yields using the pH-shift method were 23%, 6% and 25%, respectively, for the listed species. For *U. lactuca*, the yield was further raised to 29% by incorporating a pre-incubation step at pH 8.5 prior to further protein solubilisation at pH 12. The pH-shift process was also successfully used as a first step in a sequential recovery of proteins, carrageenan, pectin and cellulose from *P. umbilicalis*, showing potential as a biorefinery tool. Throughout the work, the effect of different protein analysis methods on the achieved concentrations of protein in seaweed and seaweed protein extracts was evaluated. This revealed that the analytical choice has a profound impact on the results, especially for the extracts.

To summarise, it was possible to increase the level of proteins and lipids in seaweed through alterative cultivation protocols, and the seaweed proteins could then be further concentrated using the pH-shift process. These results strengthen the possibility that seaweed biomass can be a valuable complement to terrestrial vegetarian food protein sources. If considering the seaweed PUFAs as an added value to recover along with proteins, caution must, however, be taken when it comes to pre-processing storage of the seaweed biomass; these nutrients easily degrade during storage of dried seaweeds, especially in the presence of light.

**Keywords:** Seaweed, macroalgae, proteins, unsaturated fatty acids, lipid oxidation, ascorbic acid, alterative cultivation, long-term storage, protein extraction, pH-shift, sequential recovery

## LIST OF PUBLICATIONS

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- I. Toth, G.B., **H. Harrysson**, N. Wahlström, J. Olsson, A. Oerbekke, S. Steinhagen, A. Kinnby, J. White, I. Undeland, U. Edlund, E. Albers, H. Pavia, Effects of irradiance, temperature, nutrients, and  $p\text{CO}_2$  on the growth and biochemical composition of cultivated *Ulva fenestrata*. Manuscript
- II. **Harrysson, H.**, J. Liberg Krook, K. Larsson, C. Tullberg, A. Oerbekke, G.B. Toth, H. Pavia and I. Undeland, Effect of storage conditions on lipid oxidation, nutrient loss and colour of dried seaweeds, *Porphyra umbilicalis* Kützinger and *Ulva lactuca* Linnaeus, subjected to different pretreatments. Manuscript
- III. **Harrysson, H.**, M. Hayes, F. Eimer, N.-G. Carlsson, G. B. Toth and I. Undeland, Production of protein extracts from Swedish red, green, and brown seaweeds, *Porphyra umbilicalis* Kützinger, *Ulva lactuca* Linnaeus, and *Saccharina latissima* (Linnaeus) J. V. Lamouroux using three different methods. Journal of Applied Phycology, 2018. **30**(6): p. 3565-3580.
- IV. Wahlström, N., **H. Harrysson**, I. Undeland and U. Edlund, A Strategy for the Sequential Recovery of Biomacromolecules from Red Macroalgae *Porphyra umbilicalis* Kützinger. Industrial & Engineering Chemistry Research, 2018. **57**(1): 42-53.
- V. **Harrysson, H.**, V.R. Konasani, G.B. Toth, H. Pavia, E. Albers and I. Undeland, Strategies for Improving the Protein Yield in pH-Shift Processing of *Ulva lactuca* Linnaeus: Effects of Ulvan Lyases, pH-Exposure Time, and Temperature. ACS Sustainable Chemistry & Engineering, 2019. **7**(15): p. 12688-12691.

Published paper not included in the thesis:

Larsson, K., **H. Harrysson**, R. Havenaar, M. Alminger and I. Undeland, Formation of malondialdehyde (MDA), 4-hydroxy-2-hexenal (HHE) and 4-hydroxy-2-nonenal (HNE) in fish and fish oil during dynamic gastrointestinal in vitro digestion. Food & function, 2016. **7**(2): p. 1176-1187.

Submitted manuscript not included in the thesis:

**Harrysson, H.**, B. Swolin, M. Axelsson M. and I. Undeland, A perfusion model approach to elucidate the role of blood removal for lipid oxidation and color changes in muscle of rainbow trout (*Ocorhynchus mykiss*).

## CONTRIBUTION REPORT

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**Paper I:** Hanna Harrysson (HH) participated in the design of the study, analysed the protein content, coordinated the analyses of fatty acids and wrote parts of the manuscript.

**Paper II:** HH participated in the design of the study, participated in a minor part of the experimental work, interpreted the data and was responsible for writing the manuscript.

**Paper III:** HH participated in the design of the study, performed the pH-shift experiments, analysed the composition of biomasses and protein extracts, interpreted the data and was responsible for writing the manuscript.

**Paper IV:** HH performed the pH-shift experiments, analysed the protein content and wrote the protein part of the manuscript.

**Paper V:** HH planned the design of the study, performed all pH-shift experiments and protein analyses, interpreted the data and was responsible for writing the manuscript.

## ABBREVIATIONS

---

ALA	Alfa-linolenic acid
ASE	Accelerated solvent extraction
CEAA	Conditionally essential
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
dw	Dry weight
EAA	Essential amino acids
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl esters
FTIR	Fourier Transform Infrared
HHE	4-hydroxy-trans-2-hexenal
HNE	4-hydroxy-trans-2-nonenal
HNMR	Nuclear Magnetic Resonance
HPAEC-PAD	High-Performance Anion Exchange Chromatography with pulsed Amperometric Detection
LA	Linoleic acid
LC	Long chained
LOOH	Lipid hydroperoxide
MDA	Malondialdehyde
MUFA	Monounsaturated fatty acids
n-3	Omega 3
n-6	Omega 6
PLE	Pressurised liquid extraction
PUFA	Polyunsaturated fatty acids
PV	Peroxide value
RT	Room temperature
SEC	Size-Exclusion Chromatography
sp.	Species (singular)
spp.	Species (plural)
stdv	Standard deviation
TBARS	Thiobarbituric acid reactive substances
TCEP	Tris[2-carboxyethyl]phosphine hydrochloride
TGA	Thermogravimetric analysis
ww	Wet weight

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# 1. INTRODUCTION

---

By the year 2050, the global population is estimated to be near 10 billion [1], and the food requirement is estimated by FAO [2] to be 60% higher than today. Currently, food production is responsible for up 29% [3] of the emitted greenhouse gases, half of which comes from animal-derived food production [4, 5]. According to the International Water Management Institute, food production is also responsible for 70% [6] of all freshwater used globally. In Europe, 42% of the land area is used for agriculture, and a large portion of the produced crops is used for feed production [7]. Agriculture also consumes large amounts of pesticides [8]. Beyond the environmental effects of livestock production, high meat intake has been associated with a variety of health issues such as cardiovascular diseases and type 2 diabetes [9]. Taken together, the awareness of environmental and health-related consequences of meat consumption have created an increased demand for novel vegetarian protein sources for food, something which is often referred to as the protein shift. However, based on the arguments stated above, terrestrial sources of vegetarian protein need to be supplemented with other sustainable protein sources produced via alternative routes not requiring arable land, freshwater and pesticides.

In the present thesis, it is hypothesised that seaweed could become an important contribution to the protein shift, while also contributing with unsaturated fatty acids, vitamins, and minerals. Some seaweed species contain high levels of proteins (up to 47% of the dry weight (dw)) [10], making them a promising source of vegetable protein. Aquaculture of marine seaweed could, thereby, become a good complement to land-based crops since it does not require the use of freshwater, pesticides and land [11]. A positive side-effect of cultivating seaweed is its ability to counteract eutrophication [12] and remediate carbon dioxide [13, 14]. However, aquaculture of marine seaweed in Sweden is small, despite the long coastline, and there is a need for strategies to establish cultivation techniques to produce a nutrient-rich seaweed biomass suitable for food and feed purposes.

The shelf-life of fresh seaweed is short,  $\leq 14$  days [15-17]. A challenge to having a large-scale production of food ingredients from seaweed is to find mild and cost-effective preservation and storage solutions to use before processing of the biomass. Currently, sun drying and conventional air-drying are the most common preservation techniques for seaweeds [18]. However, such handling has been shown to affect the quality negatively, e.g. by reducing ascorbic acid and unsaturated fatty acid content [19]. The unsaturated nature of seaweed lipids [20] makes them susceptible to oxidation both during drying and subsequent storage, something that also could initiate co-oxidation of vitamins and pigments. Choe and Oh [21, 22] found that lipid oxidation in dried *Porphyra* sp. correlated with decreased tocopherol, eicosapentaenoic acid (EPA) and pigment content over two weeks. However, to the best of our knowledge, no studies are published in which lipid oxidation as well as vitamin and pigment losses in dried seaweed are followed during a prolonged storage period.

Despite the relatively high protein content of seaweed, the digestibility of proteins has been described as low due to the tough polysaccharide-rich cell walls [23-29] and abundance of polyphenols [23, 24, 29, 30]. Also, to market seaweed as a protein ingredient, the proteins need to be concentrated, similar to what is done with soy [31, 32]. This applies to both food and feed settings [33]. Therefore, food-grade, scalable, and environmentally friendly techniques for extracting the proteins from the seaweed matrix are desirable. A mapping of the best process-species combinations for extracting proteins from seaweed would be of help when building

future seaweed biorefineries. Today, most extraction protocols for seaweed, both for proteins and polysaccharides, target only one compound, which leads to a major waste of biomass. A biorefinery approach with the recovery of many valuable compounds from the harvested biomass would therefore be a more sustainable approach for full seaweed utilisation.

This work has been a part of the larger Sweaweed project which has the overall aim to develop novel strains of *Porphyra umbilicalis* (red algae) and *Ulva lactuca* (green algae) and to apply novel techniques for extraction of high-value compounds. *U. lactuca* in this work refers to the northern-hemisphere *U. lactuca*, which recently was changed to *Ulva fenestrata* [34]. However, in this thesis, it will be referred to as *U. lactuca* for reasons of general understanding. This thesis has focused on seaweeds as a potential food protein source, which also has several added values such as high levels of polyunsaturated fatty acids (PUFAs), vitamins, and minerals. To broaden the variety of seaweed species for this purpose, the brown species *Saccharina latissima* has also been included for comparative purposes.

## 2. OBJECTIVES

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The overall goals of this thesis were to i) achieve a seaweed biomass high in protein and PUFA-rich lipids, ii) evaluate different strategies for retaining the quality of the seaweed raw material during drying and storage and iii) find an efficient method for recovering seaweed proteins, which also could be integrated in a sequential recovery process for seaweed macromolecules. To achieve those goals, the specific objectives were to:

- Investigate how the cultivation conditions affect the content of proteins and lipids in *U. lactuca*. (study I)
- Evaluate how drying and storage, with and without different pretreatments, affect composition, lipid oxidation, nutrient loss and the colour of *U. lactuca* and *P. umbilicalis*. (study II)
- Investigate which total protein yields can be obtained from *U. lactuca*, *P. umbilicalis* and *S. latissima* by using three different protein extraction methods: i) water extraction followed by ammonium sulphate precipitation, ii) pH-shift processing and iii) accelerated solvent extraction (ASE). (study III)
- Evaluate how the composition in terms of total proteins, amino acids, fatty acids and ash is changing from the crude seaweeds to the final protein extracts for *U. lactuca*, *P. umbilicalis* and *S. latissima*. (study III)
- Investigate how different protein analysis methods affect the achieved protein content in seaweeds and in protein extracts thereof.
- Evaluate the power of the pH-shift process as a first step in a sequential recovery of macromolecules from *P. umbilicalis*. (study IV)
- Evaluate the effect of the enzyme ulvan lyase, prolonged pH-exposure and changed time and temperature profiles on the solubility, precipitation and yield of proteins during pH-shift processing of *U. lactuca*. (study V)

### 3. BACKGROUND

---

#### 3.1 Seaweed

Seaweeds, also called macroalgae, include all multicellular macroscopic algae [35]. Most seaweeds are found in the oceans, although there are a few freshwater species. Seaweeds have a broad variety of pigments compared to plants since these are needed to accomplish photosynthesis under aquatic conditions. Based on their pigmentation, seaweeds are divided into three groups: Rhodophyta (red), Chlorophyta (green) and Ochrophyta (brown) [35].

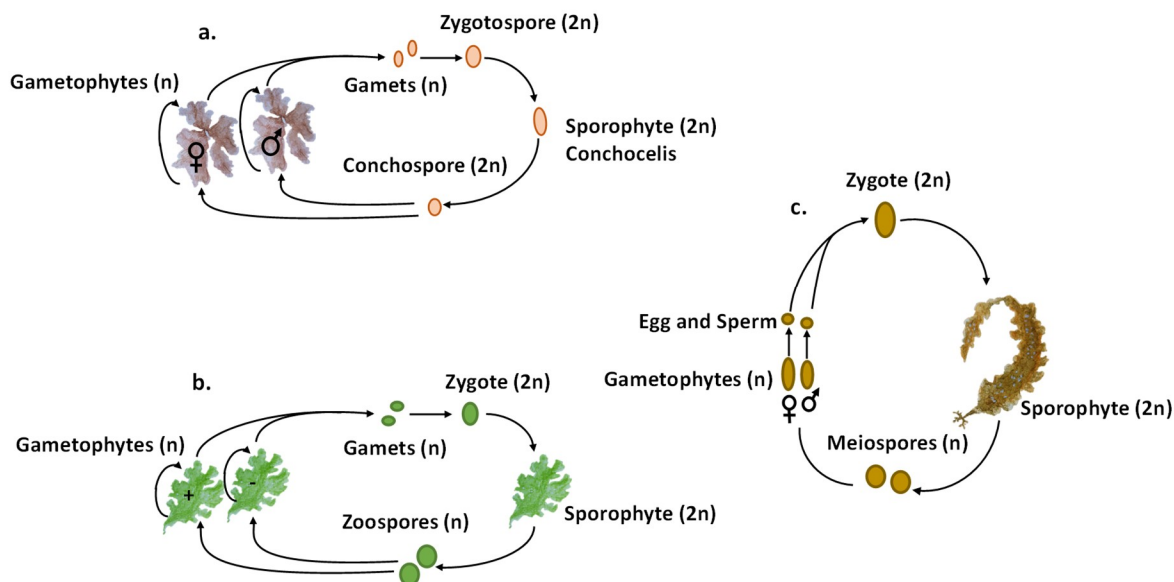
The seaweed multicellular body is called a thallus and is different from vascular plants in the sense that it lacks conducting tissues and root systems [35]. As a result, uptake of nutrients and water, as well as the photosynthesis, take place all over the algal thallus.

The basic lifecycle pattern of seaweeds alters between haploid (n) gametophytes and the diploid (2n) sporophytes [35]. Description of life cycles will be limited to the seaweed groups; within the species used in this work belong to, the red *Porphyra*, the green *Ulva*, and the brown *Saccharina*.

*Porphyra* spp. has a complicated life cycle with a microscopic stage, a filamentous diploid sporophyte, called the conchocelis stage, and a macroscopic stage, the haploid gametophyte, i.e. the thallus [36], **Figure 1**. The gametophyte (n) releases gametes (n), which merge into zygospores (2n), which in turn form the sporophyte, the conchocelis (2n). The sporophyte releases conchospores, which germinated into the young plants. However, it has also been shown that the macroscopic stage can regenerate without sexual reproduction [37], instead releasing neutral spores.

*Ulva* spp. thallus can either be gametophytes or sporophytes since those are morphologically similar and not possible to distinguish with the naked eye [35, 38]. The sporophyte (2n) releases spores which grow into the gametophyte (n), which in turn releases two types of sexual gametes, called mating types plus (+) and minus (-). The gametes merge and form new sporophytes [38], **Figure 1**. *Ulva* gametophytes can, as *Porphyra*, also reproduce asexually.

The sexually mature *Saccharina* spp. thallus forms sporangia, which releases male and female Meiospores (n), which settle and grow into microscopic gametophytes. The female gametophytes form eggs that are retained on the gametophyte, while the male gametophytes form motile sperms that are released into the water. The sperm fertilise the egg, and a new sporophyte overgrows the female gametophyte [39], **Figure 1**.



**Figure 1:** Life cycles of a) *Porphyra umbilicalis*, b) *Ulva lactuca* and c) *Saccharina latissima*.

### 3.2 Cultivation of seaweed for food use

In the past, seaweed for food use was harvested from the natural populations [40], but today, cultivation of seaweed is widely spread, with Asia dominating [41]. The yearly biomass production of seaweed was around 30 million tonnes in 2016 [42] and the five genera – *Saccharina*, *Undaria*, *Porphyra*, *Eucheuma/Kappaphycus* and *Gracilaria* – are responsible for 81-98% of this production [40, 43]. In the European countries, there is so far no, or little, cultivation and the seaweed used by the European industry comes mainly from natural populations. Different cultivation techniques, such as tank cultivation and cultivation in ponds offshore have been evaluated, finding that the former has the highest productivity per area unit but a high operational cost [41].

Seaweed aquaculture does not require freshwater or fertilisers and can remediate nutrients such as nitrogen and phosphorous [12] as well as carbon dioxide [13, 14] from the surrounding water. Hereby, seaweed cultivations can counteract eutrophication of the oceans [12, 44] and capture atmospheric carbon dioxide [13, 14]. The productivity of seaweed beds is also often higher than for terrestrial plants [45].

Both *Porphyra* spp. and *Saccharina* spp. are cultivated on a large scale, mainly in Asia [43]. For *Saccharina* spp., the seeding starts with setting of meiospores on seed strings, which later are placed on long lines off shore [43]. Due to the complicated life cycle of *Porphyra* spp., it requires a two-step cultivation [41]. In nature, the conchocelis grow in shells, something that complicates the cultivation. Simplified, cultivation starts with inserting gametophytes into tanks, whereupon they release spores. The spores then settle on shells, or another artificial substrate [37]. However, the use of free-living conchocelis is also occurring [43]. The conchocelis releases spores, which settle on nets, which later are placed offshore. To control the growth of epiphytes, *Porphyra* spp. are either desiccated or treated with acid during cultivation [43]. The finding that the macroscopic stage of *Porphyra* spp. can regenerate without sexual reproduction [37] could possibly simplify the cultivation in the future.

Even if Asia dominates seaweed cultivation, several attempts to cultivate both *Porphyra* spp. and *Saccharina* spp. have been made in the United States and in several European countries [43], indicating the growing interest in seaweed as a food raw material. Also, several efforts have been made to cultivate species beyond today's five dominating genera, such as e.g. *Ulva* species [46-48]. Several *Ulva* spp. have the ability to grow unattached, which makes them suitable for tank cultivation [49].

### 3.3 Seaweed as food

In the eastern world, seaweed has been used for food since ancient times [43]. In the western world, seaweed in its intact form is mainly used for sushi. However, there is a large industry for the production of thickening ingredients such as agar, alginate and carrageenan [50]. Beyond these hydrocolloids, seaweeds contain numerous other interesting components such as proteins, specific amino acids, lipids, vitamins, minerals and polyphenols, which provide seaweed with nutritious, sensorial and techno-functional properties. The abundance of glutamic acid in seaweed [51-54], the amino acid giving rise to the typical umami taste [55, 56], have led to an increased interest in seaweed within gastronomy [57]. Seaweed extracts have also been attributed e.g. antioxidative [58-61] and anti-inflammatory properties [62]. Further, it is suggested that specific nutrients and bioactive substances make seaweed a potential ingredient in so-called functional foods [63-65], i.e. foods with documented health effects beyond the basic nutritional properties.

#### 3.3.1 Composition of seaweed

The variation in macro composition of the three different seaweed groups (red, green and brown) can be seen in **Table 1**.

##### *Proteins and amino acids*

Seaweed can be high in proteins, up to 47% of the dw has been reported [10], see **Table 1**. Typically, the order of protein content between the three groups of seaweeds are red > green > brown [53, 54, 66-70]. A crucial factor for a new protein source is its amino acid profile. Humans can produce many of the amino acids themselves, however, some are essential amino acids (EAA), meaning that we need to get them through our food. The essential amino acids include phenylalanine, histidine, isoleucine, lysine, leucine, methionine, threonine, valine, tryptophan [71]. Seaweed, besides its relatively high protein content, also contains many or all of the EAA [23, 70, 72-75], making it a high-quality protein source. The difficulties linked to the analysis of tryptophan results in most studies not reporting the tryptophan content. However, values of 0.3-3 % tryptophan of the total protein content in seaweed have been reported in a few studies [23, 53, 73].

Besides being macronutrients, some proteins and peptides from seaweed have been reported to have bioactive properties [76, 77]. The glycoproteins lectins are carbohydrate-binding proteins which have been associated with e.g. anti-inflammatory, anti-bacterial and cytotoxic properties [76]. Phycobiliproteins play an important role in the photosynthesis of red seaweed and can constitute a major part of the cell wall in those species [76]. Some phycobiliproteins have been shown to have e.g. antitumor and anti-inflammatory properties. Due to their fluorescent properties, they are used within the biotechnology field. However, today, most phycobiliprotein for this purpose is extracted from microalgae [76].

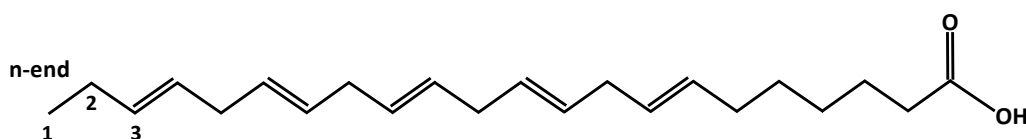


**Table 1:** Macro composition and amount of EPA and DHA reported in the literature for red, green and brown seaweed. EPA= eicosapentaenoic acid, DHA= docosahexaenoic acid.

	Red	Green	Brown
<b>Protein</b> (% dw)	<b>5-47</b> [10, 52, 70, 78-81]	<b>3-28</b> [67, 70, 73, 74, 79, 81-84]	<b>3-20</b> [52, 70, 74, 78, 80, 81, 85, 86]
<b>Lipids</b> (% dw)	<b>0.3-4</b> [20, 53, 66, 70, 80, 81, 87]	<b>0.3-8</b> [20, 66, 68, 70, 73, 74, 80, 81, 84, 87]	<b>0.3-20</b> [20, 66, 70, 74, 80, 81, 87, 88]
<b>EPA</b> (% of total fatty acids)	<b>8-59</b> [20, 53, 66, 70, 80, 87]	<b>0.8-6</b> [20, 66, 68, 70, 74, 80, 87]	<b>2-14</b> [20, 66, 70, 74, 80, 85, 87, 88]
<b>DHA</b> (% of total fatty acids)	<b>0.0-0.5</b> [20, 70, 80, 87]	<b>0.0-1.1</b> [20, 68, 70, 74, 80, 84, 87]	<b>0-13</b> [20, 70, 74, 80, 85, 87, 88]
<b>Carbohydrates</b> (% dw)	<b>27-68</b> [52, 66, 78-80]	<b>33-62</b> [66, 67, 74, 79, 80, 84]	<b>26-69</b> [52, 66, 74, 78, 80, 85]
<b>Ash</b> (% dw)	<b>7-53</b> [52, 53, 66, 70, 78-81, 89, 90]	<b>11-78</b> [66-68, 70, 73, 74, 79-81, 84]	<b>13-44</b> [52, 66, 70, 74, 78, 80, 81, 85, 86, 90]

### Lipids and fatty acids

The lipid content in seaweed is normally low, ~1-7% on a dw basis [20, 70, 81, 91]. However, as can be seen in **Table 1**, higher levels, ~16-20% of the dw, have been reported for some brown species [81]. The fatty acid profile of seaweed lipids consists of high levels of PUFAs, with levels up to 69% of the total fatty acid content being reported [91]. Seaweed can also produce lipids containing > 18 carbon, making them unique in comparison to land-based plants, which lacks this ability [92]. The long chained (LC) n-3 PUFAs eicosapentaenoic acid (EPA C20:5) and docosahexaenoic acid (DHA, C22:6), which are connected to a variety of health benefits, such as reducing inflammation and lowering the risk for cardiovascular diseases [93, 94], have both been found in seaweed. The notations n-3 (omega 3) or n-6 (omega 6) specify that there is a double bond in the fatty acid located between the third and fourth carbon or between the sixth and seventh carbon, counting from the methyl or n-end, see **Figure 2** [95]. Humans cannot synthesise lipids with double bonds positioned in the n-3 (or the n-6) position and therefore the n-3 fatty acid alpha-linolenic acid (ALA C18:3) and the n-6 fatty acid linoleic acid (LA) are essential for us [95]. ALA can be enzymatically converted in the body to EPA and DHA [95, 96]. However, the conversion rate is low and therefore direct consumption of EPA and DHA is preferred. The red species normally contain the highest levels of EPA [20, 53, 66, 70, 87, 97], **Table 1**. DHA is normally found in lower levels but has been found in red [20], green [68, 74, 84] and brown species [74, 80, 85, 88, 98], with the highest levels being found in the brown ones [85, 87]. The reported n-6 to n-3 ratios in seaweed range between 0-2.6 [70, 91]. A n-6/n-3 ratio  $\leq 1$  is considered beneficial as it can suppress cardiovascular and inflammatory diseases [99]. In the western world we often have a diet with a very high ratio of n-6/n-3 fatty acids (15/1), which is associated with the development of a variety of such diseases [99].



**Figure 2:** The n-3 PUFA Docosahexaenoic acid with the first double bond situated between the third and fourth carbon, counted from the n-end.

### *Carbohydrates*

A vast part of the seaweed biomass is made up of polysaccharides, **Table 1**. However, the types of polysaccharides differ from the ones found in terrestrial plants [100]. The diversity of polysaccharides between seaweed species is high [35] and could be divided into storage polymers and constituents of the cell wall. Respective main storage polymer in red, green and brown seaweed is floridean starch, starch and laminarin [35]. In this thesis work, however, the sulphated polysaccharides making up the cell wall of the seaweeds, such as carrageenan and agar found in red species [35, 101] and alginate found in brown species [35, 102], have been in particular focus. Those polysaccharides have gel-forming properties, which gives both rigidity and elasticity to the cell wall. The polysaccharides are also of great commercial interest [64, 101, 103-105]. Today there is large industry developed to extract carrageenan, agar and alginate from seaweed, based on their use as thickening and emulsifying agents in food and pharmaceutical products [64, 101, 104, 105].

The polysaccharides in green seaweed have not been so thoroughly studied as the polysaccharides found in red and brown seaweed species. However, for green seaweed species, the major polysaccharide in the cell wall is ulvan, which contains glucuronic acid, rhamnose, iduronic acid and xylose [106].

Besides the above-mentioned polysaccharides, cellulose can also be found in the biomass from red, green and brown seaweed [35, 107]. The main source of cellulose today is terrestrial plants [108]. However, it is suggested that some seaweed species could be used for industrial production of cellulose [107].

### *Micronutrients*

The term micronutrients includes vitamins and minerals, which are essential for human health. Seaweed contains a variety of vitamins and are especially rich in ascorbic acid, with reported levels ranging between 0.026-3mg/g dw [81, 109]. The highest values in seaweed are not far away from the ascorbic acid content in lemons (4.8mg/g dw), stated by the Swedish Food Agency [110]. Animal-derived food is today an important source of vitamin B<sub>12</sub> (cobalamin) [9]. According to the recent report by Parodi et al. [11], the B<sub>12</sub> level per gram protein is, however, much higher for *S. latissima* than for different animal-derived sources. MacArtain [109] reported levels of B<sub>12</sub> in seaweed between 0.005-0.63ug/g, where *Ulva* spp. contained the highest amounts. Other vitamins found in seaweed are e.g. B<sub>1</sub> (thiamine), B<sub>2</sub> (riboflavin) and E (tocopherol) [111, 112]. Seaweed also contain carotenoids [64] with vitamin A-activity [112].

The mineral content in seaweed is high, which is reflected by high ash content, **Table 1**. Provided that the minerals are bioavailable, seaweed could therefore be a good source of essential minerals and trace elements. The most abundant minerals are Na, K, Ca, Mg [90, 113]. The high mineral content, together with the high glutamic acid content, make seaweed interesting also from a salt-replacer perspective [114]. High iodine levels have been reported in, especially, brown seaweed species [69, 115, 116]. Iodine is essential for humans but can become toxic in high doses. Therefore, seaweeds, depending on species, could both be a valuable source for iodine but also constitute a risk for a too high iodine intake.

### 3.4 Postharvest handling of seaweed and its impact on quality

The high water content of fresh seaweed (~84-94%) [81] reduces its shelf life based on microbiological, sensorial and chemical analyses [15-17]. The reported shelf life of the fresh seaweeds *Palmaria palmata*, *Ulva rigida* and *Gracilaria* spp. when stored at 2-20 °C varied between 3-14 days [15-17]. After harvest, it is also common to rinse the seaweed in tap water, to remove epiphytes and impurities, something that can reduce the shelf life further [15]. For *U. rigida* and *P. palmata* rinsing reduced the shelf life from 14 to 3 days, based on the physical quality (colour, texture and aroma), when stored at 4 °C. The authors [15] stated that the shelf life for non-rinsed seaweeds found in their study (14 days) was longer than the one obtained in commercial handling, where the shelf life is 2-3 days. The authors speculated that this is due to the metabolic heat formed when large quantities of seaweed are stored in bulk at the manufacturing sites. Paull et al. [16] found that the average shelf life, based on the physical appearance, colour, cellular leakage and respiration rate of *Gracilaria* spp. was 4 days when stored wet at either 2 °C or 10 °C. However, a higher temperature, 15-17 °C, was found to have a beneficial effect on the physical appearance during storage but did not prolong the overall shelf life. Light enhanced the discolouration of *Gracilaria* spp., and by immersing the seaweeds in seawater and storing it in darkness, the shelf life was extended to 30 days [16]. The optimal storage temperature seems to be species-specific, since Nayyar et al. [17] instead found that the quality, based on microbial, sensory and biochemical properties, of *P. palmata* was retained better during 14 days of storage at 2 °C compared to at 7 °C, while the opposite was seen for *Gracilaria tikvahiae*. In all the above-mentioned studies [15-17], it was concluded that the microbial growth is not the shelf life-limiting factor; instead the physical appearance, as well as losses of texture, colour and cellular leakage, were the main causes for the quality degradation during storage.

To prolong the shelf life of seaweed, different techniques such as drying, salting, freezing and fermenting have been applied [15, 117, 118]. Industrially, drying, sun-drying or conventional air-drying [18], are the most applied methods to prolong the shelf life, but also to reduce the weight of the seaweed [119]. Drying can, however, negatively affect the quality of the seaweed. Sun-dried, compared to fresh, *S. latissima* contained 5-10 times less total phenolic compounds and ascorbic acid [120]. Similarly, oven-drying of *Himanthalia elongata* at 25 °C reduced the total phenolic compound and the flavonoid content by around 50% compared to the fresh one [121]. For several species, conventional air-drying at 60-70 °C has also been shown to affect the quality negatively, compared to freeze-drying, by lowering the levels of amino acids [19, 119], unsaturated fatty acids [19], total phenolics [122, 123], carotenoids [123] and ascorbic acid [19]. However, the protein content (based on the dw) was higher in oven- and freeze-dried than frozen *S. latissima* [124] and Stevant et al. [125] did not find any differences in amino acid content between freeze-dried and oven-dried (25-70 °C) *S. latissima*. Further, the odour of *S. latissima* [125] and the colour of *Ulva* spp. [123] were unaffected by the type of drying (oven- and freeze-drying) as well as the drying temperature used [125].

The physio-chemical properties of seaweed can also be affected by the drying technique. For instance, oil holding capacity and water holding capacity were highest and lowest, respectively, in freeze-dried compared to sun-dried *S. latissima* [120]. For *Sargassum* spp., swelling capacity in water, as well as water- and oil-holding capacity, were higher for the freeze-dried samples compared to the oven-dried ones [119]. Further, the swelling capacity in water was higher for freeze-dried compared to oven-dried *S. latissima*, and the authors [125] therefore concluded

that, for food applications, freeze-drying or drying at low temperatures (25 °C) is preferred. Abdollahi et al. [124] found higher protein recovery from freeze-dried than oven-dried *S. latissima* when using the pH-shift method. For *Sargassum* spp., protein extractability was instead higher for oven-dried than freeze-dried biomass and correlated with a lower phenolic content in the former one [119].

#### **3.4.1 Lipid oxidation and its occurrence in seaweed**

Lipid oxidation is a chain reaction that can be initiated by free radicals such as the hydroxyl radical ( $\bullet\text{OH}$ ), photosensitisers such as chlorophyll or enzymes such as lipoxygenases [126]. Free radical initiated lipid oxidation is often referred to as autoxidation and results in a lipid radical ( $\text{L}\bullet$ ), which quickly reacts with oxygen, forming a lipid peroxy radical ( $\text{LOO}\bullet$ ). This radical in turn extracts hydrogen from another fatty acid, forming a new lipid radical ( $\text{L}\bullet$ ) and a lipid hydroperoxide ( $\text{LOOH}$ ), which is also called primary lipid oxidation product [127].

Termination can occur when an antioxidant donates hydrogen to a free radical, forming less reactive molecules or when two radicals react with each other [127]. Lipid hydroperoxides are unstable and easily decompose further in the presence of trace metals into secondary lipid oxidation products, such as a variety of aldehydes, ketones and organic acids, which give rise to unpleasant taste and odour, often referred to as rancidity [126]. Both primary and secondary oxidation products can also react with proteins and amino acids forming protein cross-links as well as modifications on the amino acid side chains, which could change both structure and colour [126, 128-130]. During different steps of the lipid oxidation reaction, pigments, antioxidants and vitamins can also co-oxidise with fatty acids, resulting in colour loss and further loss of the nutritional value.

Despite the relatively high amount of PUFAs in seaweed lipids, as well as the presence of e.g. chlorophyll and lipoxygenase [131], lipid oxidation in seaweed is recorded in only a few studies, targeting only primary oxidation products. In dried *Porphyra* sp., development of lipid hydroperoxides and conjugated dienes correlated with a decrease in  $\alpha$ -tocopherol and polyphenols during both dark and light storage for 14 days at 40 °C [21, 22]. Light stimulated the lipid oxidation along with a decrease in polyphenols and pigments, especially chlorophyll [22]. At  $a_w \geq 0.51$ , the formation of hydroperoxides and conjugated dienes, as well as the loss of EPA, increased remarkably [21]. Although they did not analyse lipid oxidation products, Schmid et al. [132] showed that the PUFA content of freeze-dried *Laminaria digitata* and *P. palamata* was less than half of the initial value after 22 months of storage at 20 °C in darkness, indicating that lipid oxidation has occurred.

To the best of our knowledge, no one has previously analysed any specific oxidation products in seaweed, something that potentially could give valuable information about the route of lipid oxidation in this type of biomass. The aldehydes hydroxy-trans-2-hexenal (HHE), 4-hydroxy-trans-2-nonenal (HNE) and malondialdehyde (MDA) have gained increasing interest in recent years as markers for lipid oxidation [129]. HHE is derived from n-3 fatty acids, whereas HNE is specific for oxidation of n-6 fatty acids [133]. MDA, on the other hand, forms from oxidation of both n-3 and n-6 fatty acids [127]. HHE, HNE and MDA have also received attention due to their high reactivity with e.g. proteins, free amino acids and phospholipids [128] which *in vivo* can be linked e.g. to cytotoxic, genotoxic and mutagenic effects [134, 135]. HHE, HNE and MDA are known to react with the side chain of lysine, cysteine and histidine and thus induce protein carbonylation [136].

### **3.5 Use of edible coatings to improve the quality of seaweeds**

Today, edible coatings are used within the fruit industry as oxygen barriers to protect against oxidation e.g. during drying [137]. For instance, coatings better retained the ascorbic acid content in papaya [137] and pineapple [138] and the carotenoid content in pumpkin [139] during drying compared to non-treated samples. There are three different groups of coatings used for this purpose: hydrocolloids (e.g. proteins and polysaccharides), lipids (e.g. fatty acids and waxes) and composites (where different polymers are combined [140]) [141]. To the best of our knowledge, no one has earlier applied edible coatings to seaweed. However, there are reasons to believe that coatings have the potential to protect the seaweed against quality degradation during drying and subsequent storage, something which is still to be explored.

#### ***3.5.1 Whey-based coatings***

Different globular proteins, such as whey proteins, are used as coating material within the food industry [141]. Whey coatings are reported to have better mechanical and barrier properties than many other protein-based coatings such as e.g. those based on soy and wheat [142]. However, to avoid the whey coating from coming brittle and to enhance the moisture barrier properties, plasticisers such as glycerol is often added [142]. The coating is often applied by dipping, spraying, or rolling to achieve a thin wet layer on the food surface [143]. When the carrier solution evaporates and the whey denature by heating, films are formed by cross-linking of the proteins. However, films can form from native proteins too, which is why heating is not necessarily needed [143].

### **3.6 Protein recovery from seaweed**

#### ***3.6.1 Challenges***

The rigidity of the seaweed cell walls hinders protein extraction, and hence, different techniques to break the cell walls prior to protein extraction have been applied, i.e. mechanical forces, osmotic disruption and the use of cell wall acting enzymes [144-148]. The cell wall mucilage [149, 150] and the gel-forming properties of many of the seaweed polysaccharides can also reduce the seaweed protein extractability [150, 151], as well as their ability to form ionic interactions with the proteins [151]. Further, it is described how the polyphenols of seaweed, many of which can precipitate proteins [152], render extraction difficult [30, 150]. Wong et al. [122] found a correlation between phenolic content and protein extractability. Brown seaweed also contain phlorotannins, which have been shown to oxidise and form covalent bonds with proteins [153].

#### ***3.6.2 Principles of protein extraction and precipitation***

In the literature, several ways for extracting seaweed proteins are described, such as water extraction and alkaline extraction [149, 154, 155], extraction with acid [155], extraction aided by enzymes acting on the cell wall of the seaweeds [144-146, 149, 156], pulsed electric field [147, 156], high shear homogenisation [156] and mechanical press [147]. However, none of the listed studies has later recovered the extracted proteins by precipitation. This was, on the other hand, done by Galland-Irmouli et al. [24] and Garcia-Vaquero et al. [157] who used water for solubilisation followed by salting-out of the proteins using ammonium sulphate. This is here referred to as the traditional method. Ammonium sulphate is commonly used for protein precipitation due to its high solubility [158]. To minimise the contact area between the proteins and water, the proteins fold, leading to precipitation when the salt levels are high enough [158]. To remove the ammonium sulphate and dewater the precipitated protein fraction, a time-consuming dialysis step is required [157]. In the literature, different versions of the traditional

method are described for seaweed, e.g. with solubilisation at alkaline pHs and the use of 2-mercaptoethanol to break disulphide bonds as ways to further increase the yield [30, 159-161]. Protein yields reported with the traditional method range from 5-46% [30, 157, 159, 160]. However, different ways of reporting protein yields (e.g. as per ingoing protein or ingoing total weight) and the lack of definition of the stated yields make direct comparisons difficult. Also, the recovery of alkali extracted proteins using filtration followed by dialysis against water is described [161]. Using this method, the achieved protein extracts contained 86% and 62% protein from *Gracilaria* spp. and *Ulva* spp., respectively, compared to 25% and 9%, respectively, in the crude seaweeds.

Another described route for precipitating solubilised seaweed proteins is via isoelectric precipitation [148]. Thus, after separating alkali-solubilised proteins from non-solubles, the pH of the soluble phase is decreased to the isoelectric point of the proteins where the proteins are precipitated. After a centrifugation step, the proteins will end up in the pellet. This strategy is often referred to as the pH-shift method. The pH-shift method with alkaline protein solubilisation followed by isoelectric precipitation has also successfully been used for extracting proteins from fish muscle [162-165], shellfish [166] and soybeans [32]. It has also been used for protein extraction from beans [167] and micro-algae [168]. In 2017, it was applied to seaweed for the first time [148] with the addition of an initial osmotic shock to disrupt the cell walls and facilitate protein solubility. The yield of proteins when *S. latissima* was subjected to the pH-shift process was 16% based on the initial protein content of the raw material. A similar procedure has been applied to *Ulva ohnoi*, where first both aqueous and alkaline extracts were prepared, followed by precipitation of the proteins at the isoelectric point [169]. In this study, the reported protein yields were ~12-22%. Veide Vilg and Undeland [148] revealed that the low yields with the pH-shift method, when applied to *S. latissima*, primarily were due to low yields in the precipitation step rather than the extraction step. Ways of increasing the precipitation by food-grade methods should therefore be developed.

Based on the massive interest in vegetarian proteins, completely new strategies for recovering proteins should be evaluated. One possible principle would be to removed lipids, phlorotannins and carbohydrates via accelerated solvent extraction (ASE), also referred to as pressurised liquid extraction (PLE) [170]. In this technique, solvents are used under both high temperature and pressure [171], decreasing surface tension of the solvents to more easily penetrate the tissue or matrix to be extracted. ASE is fast and demands little solvent compared to other extraction methods [170]. This technique has so far not been used for seaweed protein extraction but could have potential to be tested.

### **3.7 Protein analysis methods for seaweed**

There is a variety of methods for quantitative protein determination, and it is well known that the method of choice has an impact on the results [172]. A common method for estimating protein content in seaweed is based on analysis of the total nitrogen. Thereafter, the total protein content is calculated based on a nitrogen-to-protein conversion factor. A common factor used is 6.25. For seaweed, it has, however, been shown that using this factor overestimates the protein content [173], and instead, a universal nitrogen-to-protein conversion of 5 has been suggested by Angell et al. [173]. Colorimetric methods, like Lowry [174], are both sensitive and fast, however, the result can be affected by interfering substances [175]. Amino acid analysis is the most time-consuming protein analysis method. However, amino acid analysis is the only protein measurement method that is not affected by interfering substances, such as

pigments [172] and non-protein nitrogen. Analysis of the total amino acid content is also used as a reference method when compiling nitrogen-to-protein conversion factors [72, 75, 82, 176].

## 4. METHODS AND METHODOLOGY CONSIDERATIONS

### 4.1 Study design

This thesis is based on five studies (papers I-V). An overview of the different studies is seen in Figure 3.

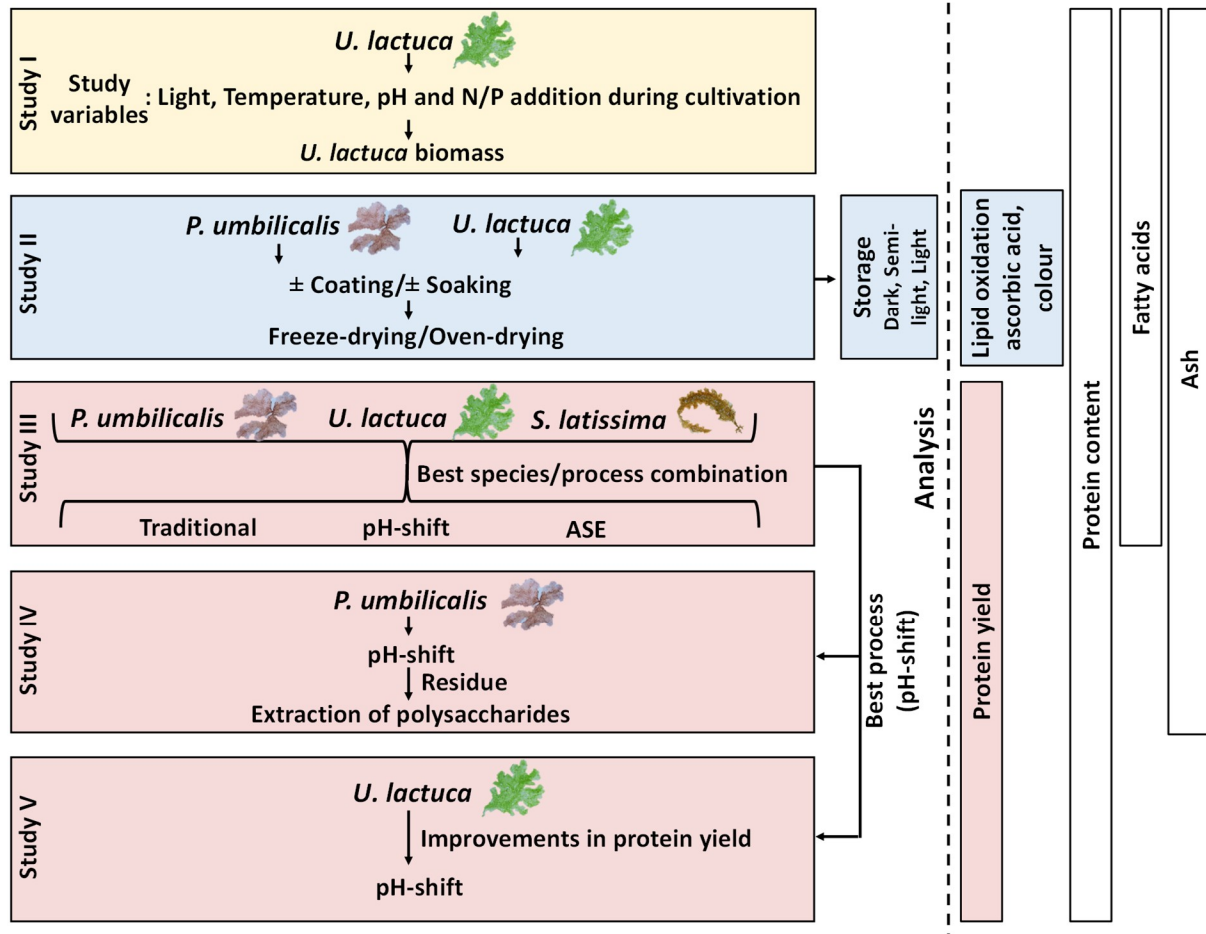


Figure 3: Schematic overview of studies I-V.

To evaluate the cultivation conditions in relation to the content of proteins and lipids in *U. lactuca*, alternative cultivation experiments were performed in study I.

In study II, the effect of the different pretreatments post-harvest, drying and storage on composition, lipid oxidation, nutrient loss and colour was evaluated for *P. umbilicalis* and *U. lactuca*. The seaweeds were subjected to different pretreatments: (i) soaked in freshwater and (ii) coated using a whey-based material prior to oven-drying. Dried samples were then stored under varying light conditions. During storage, the quality change was followed by measuring the lipid oxidation products MDA, HHE and HNE, fatty acids, ascorbic acid and colour.

In study III, the efficiency of the traditional method, the pH-shift method and the ASE method in terms of total protein yield and the composition of the achieved extracts was evaluated for *P. umbilicalis*, *U. lactuca* and *S. latissima*.

In study IV, the most promising protein extraction method from study III, the pH-shift process, was evaluated as an initial extraction step in a sequential recovery of macromolecules from *P.*



*umbilicalis*. The residue from the pH-shift process was used for further extraction of carrageenan, pectin and cellulose.

In study V, strategies to improve the protein yield during the pH-shift process for *U. lactuca* was investigated. The strategies evaluated were: i) addition of the enzyme ulvan lyase, ii) prolonged incubation at pH 8.5 and iii) different temperatures.

## 4.2 Seaweed raw material

All seaweeds used in this work were cultivated at the Sven Loven Center for Marine Infrastructure at Tjärnö, Sweden (58°52'33.2724''N, 11°8'47.202''E). *U. lactuca* and *S. latissima* were both fully cultivated in indoor tanks. For *P. umbilicalis*, turfs were collected for study II at Tjärnö (58°52'33.272''N, 11°8'47.202''E), and for study III at Inre Vattenholmen (58°52.622N; 11°6.876E), rinsed to remove epiphytes and further tank-cultivated.

In study I, light intensity, temperature, pCO<sub>2</sub> and the addition of nitrate and phosphate were varied to evaluate the effect of those parameters on growth rate and the intracellular concentration of protein, lipids and carbohydrates in *U. lactuca*. In studies II-IV, seaweeds were grown under a neutral light cycle (16 h daylight, 8 h darkness) in a light intensity of 50 μM photons m<sup>-2</sup>s<sup>-1</sup> at 12 °C. During cultivation, the seaweeds received continuous filtered (1 μm) seawater without additional medium. The same biomass was used in studies III and IV. In study V, the light intensity used was 140 μM photons m<sup>-2</sup>s<sup>-1</sup>; otherwise, the conditions were the same as in study II-IV.

After harvest, all seaweeds were dried (oven-dried in studies I and V and freeze-dried in studies III and IV) and ground using a coffee grinder. For study II, where drying *per se* was evaluated, both freeze-dried and oven-dried samples were prepared. In this study, the seaweeds were stored whole and milled after the storage. For all studies (I-V), the milled samples were kept at -80 °C until further process or analysis.

### 4.2.1 Species used in the different studies

The cultivation experiments in study I were performed on *U. lactuca* alone. This was because *P. umbilicalis* could not be kept alive during tank cultivation for a prolonged time and eventually collapsed, probably due to the need for that species to dry out regularly.

Study II comprised both *P. umbilicalis* and *U. lactuca*, since their lipid composition, as well as pigments, vary a lot, and hence they were therefore expected to respond differently, both during drying and storage.

To evaluate how seaweeds from the three main groups red, green and brown, responded to different protein extraction methods (the traditional method, the pH-shift method and the ASE method) the brown species *S. latissima* was also included in study III (together with *P. umbilicalis* and *U. lactuca*). Even if the protein content in *S. latissima* is normally lower than in *P. umbilicalis* and *U. lactuca*, the productivity of biomass per area is high [177, 178], making *S. latissima* a promising alternative for protein production, in addition to the well-established alginate production [64].

*P. umbilicalis* was used in study IV for a sequential recovery of proteins, carrageenan, pectin and cellulose, since the results from study III, showed that *P. umbilicalis* was the species resulting in the protein extract with highest protein concentration. Further, *P. umbilicalis* is rich in carrageenan, an already commercial polysaccharide [64, 101].

The pH-shift process was further improved for *U. lactuca* in study V, based on the results in study III, where *U. lactuca* formed an extract high in protein, but where the total protein yield was low. Further, it was shown in study I that the protein content in *U. lactuca* could be elevated through alternative cultivation.

### 4.3 Cultivation conditions during alternative cultivation of *U. lactuca* (study I)

It is known that the content of e.g. proteins, lipids and carbohydrates in seaweed varies over season [69, 78, 79, 85] depending on the temperature, light and nutrients available. Also, the increasingly common formed green tides of e.g. *Ulva* spp. are explained by the increased nutrients in coastal waters [179, 180]. To vary these parameters can hence be a strategy to optimize the cultivation of *U. lactuca*. Generally, increased temperature has been seen to increase both the protein and lipid content in *Ulva* spp. [180-183]. Further, the protein and lipid content have been seen to increase by increased levels of available nitrate and phosphate [184].

In study I, three experiments were performed where the effects of: i) light intensity and temperature, ii) nitrate and phosphate levels and iii) pCO<sub>2</sub> on the biochemical composition and growth of *U. lactuca* was investigated. The controlled conditions in those experiments were ambient nutrient levels (nitrate < 5 µM, phosphate < 1 µM), light intensity=50 or 110 µmol photons m<sup>-2</sup>s<sup>-1</sup>, pCO<sub>2</sub>≈ 400 ppm and 13-14 °C. The lower light intensity (50 µmol photons m<sup>-2</sup>s<sup>-1</sup>) was applied in the pCO<sub>2</sub> experiment.

Most reported light conditions during cultivation of *Ulva* spp. range from 40 to 100 µmol photons m<sup>-2</sup>s<sup>-1</sup> [180-182, 185-191], however, both higher (up to 1000 -1200 µmol photons m<sup>-2</sup>s<sup>-1</sup>) [192, 193] and lower levels (10-15 µmol photons m<sup>-2</sup>s<sup>-1</sup>) [184, 185] have been reported. In study I, two of the chosen light conditions, 50 and 100 µmol photons m<sup>-2</sup>s<sup>-1</sup>, lay within the most-used range. To evaluate the effect of a slightly higher light intensity on the growth rate and intracellular composition, 160 µmol photons m<sup>-2</sup>s<sup>-1</sup> was also investigated. Reported temperatures used in *Ulva* spp. cultivation vary a lot, between 5-32 °C, depending on the locality of the cultivation [180-182, 184-196]. Here, we evaluated 13 and 18 °C, to mimic realistic water temperatures along the Swedish coast. Nitrogen is commonly added in the form of nitrate, and here 150, 500 µM and the ambient level (< 5 µM) were evaluated. Two levels of phosphate, 50 µM and the ambient level (< 1 µM) were evaluated. The pCO<sub>2</sub> was lowered and raised, respectively, compared to the ambient level (400ppm) to 200ppm and 2500ppm, respectively. This corresponded to ~pH 8.1, 7.7 and 9.0, respectively. Most reported pH levels investigated for *Ulva* species vary between ~7.5-8.3 [180-182, 185, 189, 190, 192, 195].

### 4.4 Postharvest handling and storage of seaweeds (study II)

For an overview of all pretreatments, drying alternatives, and storage conditions applied to *P. umbilicalis* and *U. lactuca* in study II, see Table 1 in paper II.

#### 4.4.1 Pretreatments prior to drying

Rinsing seaweed biomass with freshwater to remove impurities and epiphytes is a common pretreatment in the seaweed industry [15]. The use of freshwater can induce an osmotic shock, which disrupts the seaweed cells. This has, as already mentioned, been reported to affect negatively the quality of wet seaweed [15]. We hypothesise that this also could affect the final quality of dried seaweeds. To test this hypothesis, *P. umbilicalis* and *U. lactuca* biomasses were soaked in freshwater prior to drying. Non-soaked seaweeds were used as control. Soaking instead of rinsing was applied to be able to compare this treatment with the coating treatment.

According to the suppliers of the whey coating, the coating should be dissolved in freshwater. However, to also investigate whether the type of water used for the coating solution had any effect on the final quality, the whey coating was dissolved in both fresh- and saltwater before the seaweeds were merged into the solutions.

#### **4.4.2 Drying temperatures**

In previous studies addressing the effect of drying on seaweed quality, the used temperatures vary between 25-80°C [19, 119-121, 123, 125, 197, 198]. In study II, we dried the seaweed at 40 °C based on the sensitivity of both ascorbic acid and unsaturated lipids to elevated temperatures, and further to avoid negative changes in the protein fraction, such as formation of di-sulphide bonds. Also, the supplier of the whey-based coating suggested 40 °C as an appropriate drying temperature. Since several earlier studies have reported negative effects on the quality of seaweed from oven-drying [19, 119, 122, 123], freeze-drying was included as a control in study II.

#### **4.4.3 Storage conditions for dried seaweeds**

In study II, storage of the dried seaweeds was done in room temperature (RT) to mimic the storage conditions used by a supplier, in supermarkets, or by consumers after purchase.

The effect of light on the quality of seaweed during storage is, to date, not well studied and therefore we evaluated different degrees of light during storage of the dried *U. lactuca* and *P. umbilicalis*. Our hypothesis was that light would enhance both lipid oxidation and the degradation of ascorbic acid. At the start of the storage, complete darkness and semi-light conditions were used. The latter were obtained in a room with windows and with occasional extra light from lamps. No constant light intensity was thus achievable. To fully investigate our hypothesis, a portion of the samples was therefore moved after 90 days from the dark storage to a locality without windows, where light (869±9 LUX) was turned on for 24 hours. This resulted in three storage conditions: dark, semi-light and light (after first 90 days of dark storage).

### **4.5 Methods to determine the seaweeds quality during storage (study II)**

#### **4.5.1 Determination of lipid oxidation products**

There are several methods for analysing lipid oxidation. The primary oxidation products, i.e. the LOOH, can be analysed colorimetrically after reaction with ferric thiocyanate [199], referred to as the peroxide value (PV). Carbonyls can also be analysed colorimetrically after reaction with thiobarbituric acid [200], as a measurement of secondary oxidation products. The method is referred to as the thiobarbituric acid reactive substances (TBARS) test. Both PV and TBARS are among the most common measures of lipid oxidation in foods. However, the strong pigments in seaweed make many colorimetric methods incompatible with this kind of biomass. In study II, the specific oxidation-derived aldehydes MDA, HHE and HNE were therefore analysed by an LC-MS method developed in-house [201]. Analyses were done directly after drying as well as regularly during the storage. It is important to stress that this method analyses free aldehydes and hence, aldehydes that have bound to e.g. proteins were not detected.

#### 4.5.2 Determination of fatty acids

Fatty acids of dried seaweeds were directly trans-esterified based on the earlier work on microalgae by Cavonius et al. (2014). The fatty acid methyl esters (FAME) were further analysed using GC-MS. Direct transesterification requires less solvent and is faster than the more traditional method, which first extracts the lipids using chloroform and methanol according to e.g. Bligh and Dyer [202] or Folch et al. [203] followed by esterification. In study II, fatty acids were analysed both before and after one year of storage, to follow tentative degradation.

#### 4.5.3 Determination of ascorbic acid

Ascorbic acid is very sensitive and can easily be degraded in e.g. presence of light, oxygen and metal catalysts as well as by elevated temperature and pH changes [137]. Ascorbic acid becomes oxidised in a two-step reaction, first to dehydroascorbic acid and then further to 2,3-diketogulonic acid, see **Figure 4**. The first step is reversible, and dehydroascorbic acid can be reduced back to ascorbic acid.

Ascorbic acid has an important role in lipid oxidation since it can work both as an antioxidant by scavenging of radicals, often in synergy with  $\alpha$ -tocopherol, but also as a pro-oxidant by its ability to reduce low molecular weight metal ions, e.g.  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  [204].  $\text{Fe}^{2+}$  is strongly potent in creating the very reactive hydroxyl radical through the Fenton reaction [205], and the reaction is enhanced at low pH. At low ascorbic acid concentrations, the capacity to reduce metal ions is superior to the radical scavenging ability [206].

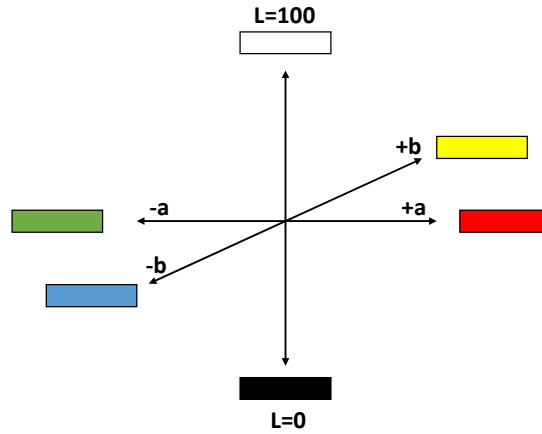
There are several colorimetric methods for analysing ascorbic acid, e.g. based on the capacity of ascorbic acid to reduce ferric chloride, which in turn reacts with potassium ferricyanide and forms a blue complex [207]. Another method is based on the oxidation of ascorbic acid to dehydroascorbic acid by a copper reagent in an ammonium-containing medium, also here resulting in a blue complex [208]. Also, a variety of commercial kits are available for colorimetric ascorbic acid determination. Again, however, the strong pigments in seaweed make them incompatible with most of the available colorimetric methods which is why we applied an HPLC-based method in study II. Here, ascorbic acid was stabilised in metaphosphoric acid to prevent it from oxidation during sample preparation. Tris[2-carboxyethyl]phosphine hydrochloride (TCEP) was then added to reduce potential dehydroascorbic acid back to ascorbic acid [209, 210] before the samples were analysed using HPLC followed by electrochemical detection, as described by Lykkesfeldt [209]. The analyses were done directly after drying and regularly during the storage, until day 230.



**Figure 4:** The oxidation of ascorbic acid to dehydroascorbic acid and further to 2,3 diketogulonic acid.

#### 4.5.4 Determination of colour

Colour was analysed directly after drying and during storage of *U. lactuca* and *P. umbilicalis* in study II, using the Hunter scale. The L-value represents the whiteness/blackness of the samples. An L-value of 100 represents total white and an L-value of 0 total black. The a-value represents redness/greenness and the b-value yellowness/blueness. The a-value and b-value have no numerical limits, instead, positive values indicate red and yellow colour and a negative values green and blue colour, see **Figure 5**. Total colour change,  $\Delta E$  was calculated according to Nayyar et al. [17], see **Equation 1**.



**Figure 5:** The Hunter scale used for colour measurements.

**Equation 1:**  $\Delta E = \sqrt{(L_2 - L_1)^2 + (a_2 - a_1)^2 + (b_2 - b_1)^2}$  \*

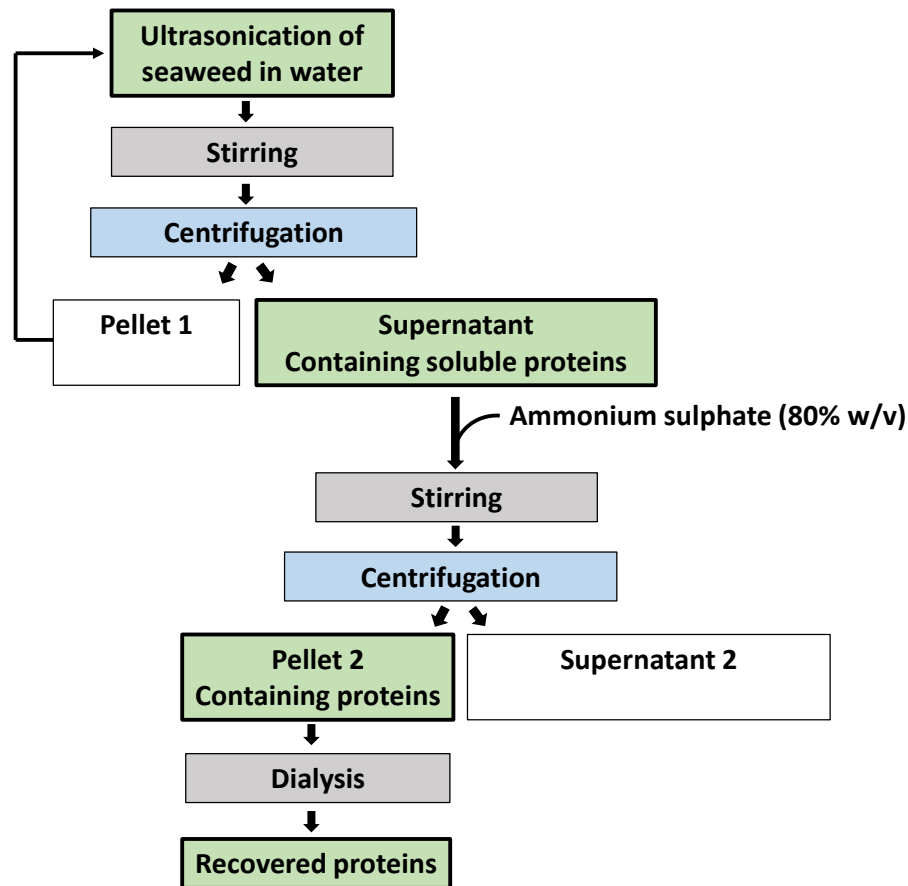
\*1 represents the value at day zero and 2 represents the value at respective analyse point.

## 4.6 Methods for recovery of seaweed proteins (studies III-V)

In study III, three different techniques were used for recovery of the seaweed proteins: i) the more traditional method with water extraction and ammonium sulphate precipitation, ii) the pH-shift process, commonly used for extracting proteins from e.g. soybeans or fish muscle, and iii) the ASE technique, which has not previously been used for seaweed protein extraction.

### 4.6.1 The traditional method

The traditional method, as earlier described by Galland-Irmouli [24], with solubilisation of the proteins in water followed by salting-out using ammonium sulphate, was applied in study III. This method [24, 157] or variations of it [30, 159-161] has been used several times for protein recovery from seaweed but not for *P. umbilicalis* and *S. latissima*. To aid the extraction of proteins into water, sonication was used, and centrifugation was applied to remove non-solubles, **Figure 6**. The supernatant was saved, and the pellet subjected to a second extraction, and the achieved supernatant was pooled with the first one. Ammonium sulphate (80% w/v) was then added to the pooled supernatants followed by stirring and centrifugation. The recovered pellet was dialysed against Milli-Q water to get rid of the ammonium sulphate.



**Figure 6:** Schematic picture over the traditional method used for protein extraction from *Porphyra umbilicalis*, *Ulva lactuca* and *Saccharina latissima* in study III. Green boxes indicate the fractions of the extractable proteins.

#### 4.6.2 The pH-shift process

The pH-shift method, with the addition of an initial osmotic shock, was recently shown to be a promising method for extracting proteins from *S. latissima* [148] and was therefore chosen to be applied in study III. The process has also been scaled up with success for e.g. soybean and fish and is Generally Regarded as Safe (GRAS), which is important in order to be applied in human food production.

Since the method had not previously been applied to either *P. umbilicalis* or *U. lactuca*, the method had first to be adapted to those species (study III). The protocol described by Veide Vilg and Undeland for *S. latissima* was used as a basis [148], see **Figure 7**. Initially, the seaweeds were homogenised in distilled water (homogenate). Thereafter, the proteins were solubilised at alkaline conditions for 20 minutes, followed by centrifugation to recover the solubilised proteins into the supernatant (supernatant 1), while insoluble material precipitated into a pellet (pellet 1). In the third step of the process, the pH of supernatant 1 was decreased to the isoelectric point of the proteins. After a second centrifugation step, the proteins were recovered into the pellet (pellet 2).

The adaption of the pH-shift protocol to *P. umbilicalis* and *U. lactuca* included finding the optimal pHs for protein solubilisation and precipitation for the two species. The adaption of the process was done using 4 volumes of water based on the dw of each of the two species, which was the same amount of water used as when the process was adapted to *S. latissima* [148]. Also, according to Veide Vilg and Undeland [148], the homogenisation of the seaweed in distilled water was followed by an incubation period (1h), exerting an osmotic shock which disrupts the cell walls. Solubility maxima (within the pH-range 2-13) and precipitation maxima (within the pH-range 1.5-8 for *P. umbilicalis* and 1.5-7 for *U. lactuca*) were then investigated. The protein solubility and precipitation at respective pH were calculated according to **Equations 2-3**. Based on the conclusion of Veide Vilg and Undeland [148] that the protein yield increases with increased amounts of water in the solubility step, a higher ratio of water was investigated also for *P. umbilicalis* and *U. lactuca* (6 volumes vs. 4 volumes). To increase the yield further, a freeze-thawing step was added between the adjustment of supernatant 1 to pH 2 and the second centrifugation step in order to stimulate precipitation of proteins. The effect of freeze-thawing was also investigated for *S. latissima*.

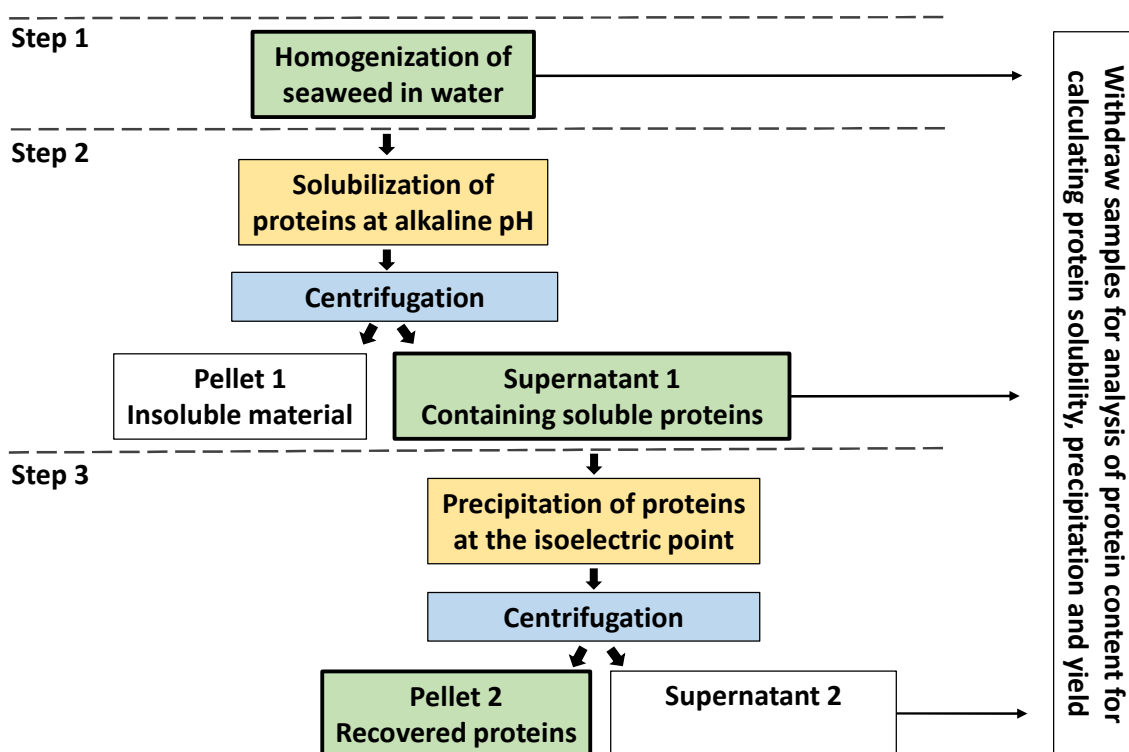
Different terminology in the literature concerning protein extraction could lead to confusion. As mentioned earlier, many have done protein extraction with no further recovery of the extracted proteins [144-147, 149, 154-156]. The extractability of proteins in many studies could therefore be compared to what in this thesis work is referred to as the solubility (**Equation 2**) or yield 1 (**Equation 7**) during the pH-shift process, depending on how the calculations were done in the specific study.

$$\text{Equation 2: Protein solubility} = 100 * \left( \frac{\text{protein concentration supernatant 1}}{\text{protein concentration homogenate}} \right)$$

$$\text{Equation 3: Protein precipitation} = 100 * \left( 1 - \left( \frac{\text{protein concentration supernatant 2}}{\text{protein concentration supernatant 1}} \right) \right)$$

In study IV, the adapted pH-shift process from study III was applied to *P. umbilicalis* followed by a series of extractions of the polysaccharides: carrageenan, pectin and cellulose (section 4.7).

In study V, the pH-shift process was further improved for *U. lactuca*. A recent study [211] described a novel ulvan lyase from the bacteria *Formosa agariphila*, which was overexpressed in a strain of the bacteria *Escherichia coli* and subsequently purified. The ulvan lyase enzyme is specific against ulvan, which is the major polysaccharide in *U. lactuca* [106]. By applying the ulvan lyase in the first step of the pH-shift process, it was hypothesised that the enzyme should hydrolyse the ulvan in the *U. lactuca* cell wall and hence aid the protein solubility. To adjust the process conditions to the use of enzymes, also the effect of an extract incubation step at pH 8.5 during the first solubility step and different process temperatures were examined.



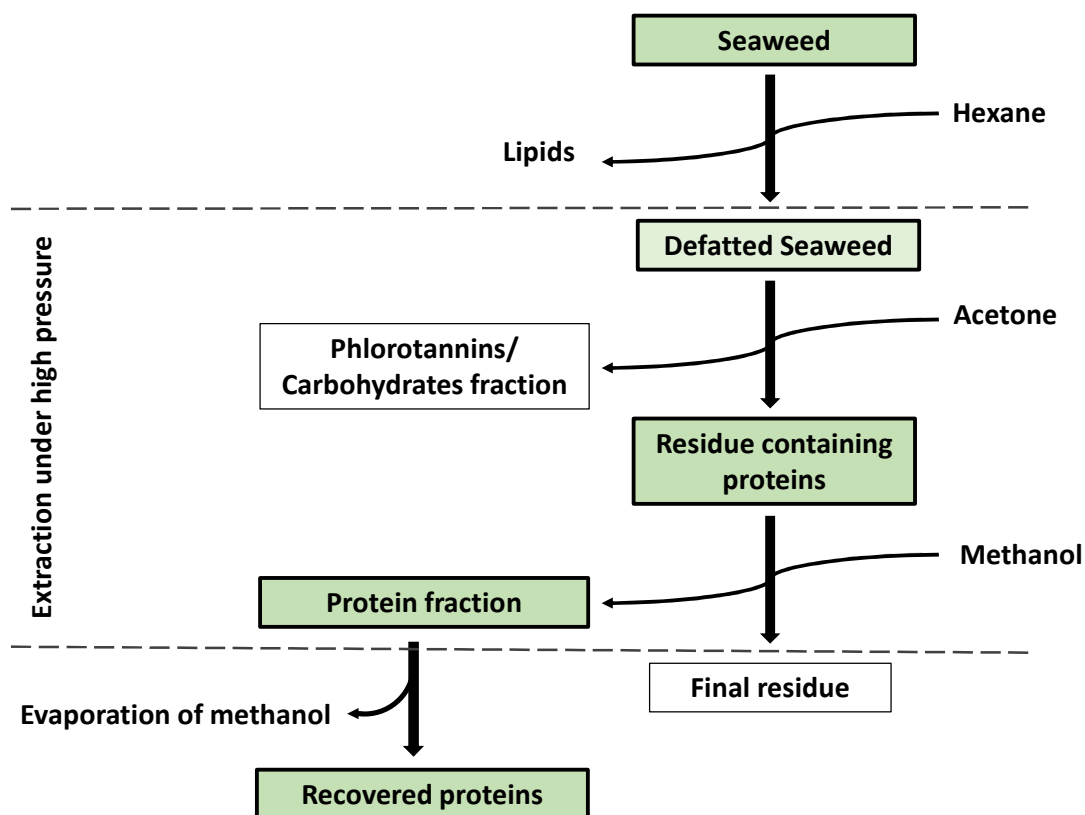
**Figure 7:** Schematic picture over the pH-shift process. The figure is based on the procedure described by Veide Vilg and Undeland [148] which was used when adapting/improving the process for *Porphyra umbilicalis*, *Ulva lactuca* and *Saccharina latissimia* in study III. Green boxes indicate the fractions of the extractable proteins.

#### 4.6.3 The accelerated solvent extraction (ASE) method

The ASE method has earlier been used for extraction of e.g. fucoxanthin [212] and polyphenols [213, 214] from seaweed, but not proteins. The idea behind using it for protein extraction in study III was the possible advantage of a sequential recovery of lipids and proteins. Also, the possible separation of phlorotannins from *S. latissimia* before protein recovery could have a positive impact on the digestibility of the achieved protein extract. Food-grade solvents could be used during the process, and the method produces little waste [171]. Before protein extraction, the seaweed samples were de-fatted using hexane, after which carbohydrates and



phlorotannins were extracted together using ASE with 70% acetone. The residual was extracted for proteins using ASE with 50% methanol as solvent, **Figure 8**.



**Figure 8:** Schematic picture of the ASE method, which was used for extracting proteins from *Porphyra umbilicalis*, *Ulva lactuca* and *Saccharina latissimia* in study III. Green boxes indicate the fractions of the extractable proteins.

#### 4.6.4 Calculation of achieved protein yield during the recovery processes

In the literature, yields from seaweed protein extractions are commonly reported in three different ways. The first way is weight yield (**Equation 4**), not taking the protein concentration of the starting material or extract into account. The second way is using the amount of extracted proteins divided by the amount of protein present in the raw material from the start (**Equation 5**), i.e. the true protein yield, sometimes also referred to as percent recovered proteins. A third way is to divide the absolute amount of protein extracted by the weight of the starting material (**Equation 6**). Since the protein concentration in the achieved extracts was of high importance in study III, and since study IV focused on how much of the initial seaweed proteins we could recover, we have calculated the true protein yield, according to **Equation 5**. However, during adaption of the pH-shift process protocol to *U. lactuca* and *P. umbilicalis* in study III and during improvements of the process for *U. lactuca* in study V, we chose to use **Equations 7-9** to calculate the true protein yield. This was because we thereby could base the yield calculations on protein measurements of the supernatants. The different ways of reporting protein yields, as well as the lack of definitions of the terms ‘yield’ and ‘protein yield’ in studies on protein extraction often make direct comparisons between studies difficult.

$$\text{Equation 4: Weight yield} = 100 * \left( \frac{\text{amount dry extract}}{\text{amount dry seaweed}} \right)$$

$$\text{Equation 5: Protein yield} = 100 * \left( \frac{\text{concentration protein in dry extract} * \text{amount dry extract}}{\text{concentration protein in dry seaweed} * \text{amount dry seaweed}} \right)$$

$$\text{Equation 6: Extraction yield} = 100 * \left( \frac{\text{absolute amount protein extracted}}{\text{amount dry seaweed}} \right)$$

$$\text{Equation 7: Yield step 1} = 100 * \left( \frac{\text{protein concentration supernatant 1} * \text{volume supernatant 1}}{\text{protein concentration homogenate} * \text{volume homogenate}} \right)$$

$$\text{Equation 8: Yield step 2} = 100 * \left( 1 - \left( \frac{\text{protein concentration supernatant 2} * \text{volume supernatant 2}}{\text{protein concentration supernatant 1} * \text{volume supernatant 1}} \right) \right)$$

$$\text{Equation 9: Protein yield} = 100 * \left( \frac{\text{yield step 1}}{100} * \frac{\text{yield step 2}}{100} \right)$$

## 4.7 Seaweed polysaccharide extraction methods (study IV)

The process used today [215] for extracting carrageenan from red seaweed is harsh, using alkaline extraction (pH 8-9) at high temperature (80-95 °C) for several hours (2-4h). Through this process, other valuable compounds, such as proteins, are destroyed and the residue is discarded. Study IV aimed to make a smart sequential recovery of macromolecules from *P. umbilicalis*, without destroying their functionalities. Carrageenan was therefore extracted from the insoluble fraction from the first step of the pH-shift process, using Na<sub>2</sub>CO<sub>3</sub> at pH 9.5 at 90 °C for 4 h. The residual insoluble fraction from the carrageenan extraction was thereafter extracted for pectin using HCl at pH 2 at 90 °C for 4 h. Before recovery of the cellulose fraction, lipids were removed. To make this step more environmentally friendly, toluene and ethanol were used instead of the more traditional Bligh and Dyer method [202], with methanol and chloroform. Cellulose was then recovered using a method earlier applied to seaweed [107] by using NaOH at 80 °C for 24 h. The insoluble fraction was further boiled in HCl for 30 min and thereafter stirred at 60 °C for 24 for. The remaining insoluble fraction was recovered as the cellulose fraction.

## 4.8 Analysis methods to determine the composition of seaweeds and seaweed extracts (studies I-V)

### 4.8.1 Protein measurement methods (studies I-V)

Protein content was analysed in this thesis using three different principles: i) as total amino acids, ii) colorimetrically by the Lowry method [174] and iii) as total nitrogen using combustion, based on the Dumas-principle, and a modified version of Kjeldahl [216].

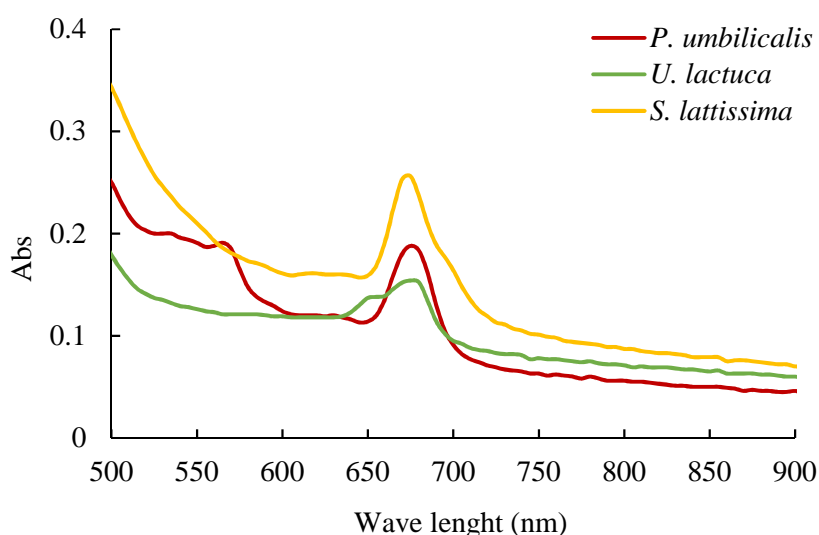
#### *Amino acids*

The quality of a protein extract is highly dependent on the amino acid profile and therefore amino acids were analysed both in the seaweed biomasses and the achieved extracts in study III. Prior to analysis using LC-MS, amino acids were hydrolysed under acidic conditions. Tryptophan is degraded during acidic hydrolysis [73, 217] and was therefore not possible to detect. The few reported values on tryptophan in seaweed range between 0.3-3% of the total protein content [23, 53, 73] and thus this amino acid constitutes a minor fraction.

#### *The Lowry method*

Total protein content in the seaweed biomasses and the seaweed protein extracts was determined according to the method of Lowry et al. [174] as modified by Markwell et al. [218]. The method measures only soluble proteins and therefore a pretreatment step to solubilise the non-soluble proteins was necessary. Veide Vilg and Undeland [148] showed that boiling in SDS compared to homogenisation in an alkaline solution (0.1M NaOH) at RT, both followed by centrifugation, gave the highest protein levels for *S. latissima* when using Lowry determination. It was also shown in that study [148] that this pretreatment gave the values closest to the values achieved using Kjeldahl. Therefore, this pretreatment was also evaluated for *P. umbilicalis* and *U. lactuca*. The result, however, revealed that boiling in SDS had no effect on the total protein content of these species compared with alkaline homogenisation. Rather, the centrifugation step applied after both the SDS and alkali treatments resulted in remarkably lower total protein levels compared to when no centrifugation was applied. Therefore, homogenisation with 0.1 M NaOH, without any centrifugation, was chosen as the pretreatment step prior to Lowry determination in this work.

Despite that the Lowry method has been used since 1951 [174], it is not fully understood. However, it is described that during the first step,  $\text{Cu}^{2+}$  reacts with the peptide bonds in peptides/proteins containing three or more amino acids, forming a coloured complex. During the second step, the tungsten molybdenum in the Folin-Ciocalteu reagent is oxidised and forms a blue complex. During this process, the earlier formed copper complexes can be reduced. However, some specific amino acids – tyrosine, tryptophan and cysteine – can also work as electron donors [219, 220]. Therefore, the achieved result is dependent on the type of amino acids in the sample. Since it is a colorimetric method, the results may also be affected by interfering substances [175, 220]. To avoid interference from the chlorophyll of the seaweeds, which has a clear absorption peak at 665nm, absorbance readings were done at 750 nm instead of 660 nm, which previously was suggested by Markwell et al. [218]. To ensure that no other pigments in the seaweeds would interfere with the blue complex at 750 nm, a scan in the range 500-900 nm was recorded for each seaweed species after homogenisation in 0.1M NaOH, see **Figure 9**. Also, since the Folin-Ciocalteu reagent is used to analyse phlorotannins [221], it could affect the protein result for brown seaweeds which could be rich in phlorotannins [222].



**Figure 9:** Scan of alkaline extracts of *Porphyra umbilicalis*, *Ulva lactuca* and *Saccharina latissima* between 500 and 900 nm. No interference at 750 nm is visible.

Due to the speed and sensitivity of the Lowry method, it was used when analysing the multiple samples emerging during the adaption of the pH-shift process to *U. lactuca* and *P. umbilicalis* in study III and also during the improvements of the pH-shift method in study V. Further, it was used for analysing the total protein content in seaweed biomasses and achieved extracts in study III. The latter enabled a comparison with the data obtained from amino acid and nitrogen analyses and thereby render conclusions on e.g. the effect of phlorotannins in the Lowry method.

### *Total nitrogen*

Two different methods, the Kjeldahl method and the Dumas method, were used for analysing total nitrogen in this thesis. Differences between the methods lie particularly in the fact that Kjeldahl measures only organic nitrogen while the Dumas technique measures all nitrogenous compounds [220]. Therefore, when using the same nitrogen-to-protein conversion factors for these two methods, the Dumas method has been reported to yield 1.4% higher protein content in foods compared to the Kjeldahl method [223].

The Dumas method analyses total nitrogen through combustion. The sample is combusted in the presence of oxygen, forming  $\text{H}_2\text{O}$ ,  $\text{N}_2$ ,  $\text{NO}_2$ ,  $\text{SO}_2$  and  $\text{CO}_2$  [224].  $\text{H}_2\text{O}$ ,  $\text{SO}_2$  and  $\text{CO}_2$  are absorbed, while  $\text{NO}_2$  is reduced to  $\text{N}_2$  by copper before nitrogen is analysed by a thermal conductor. In this study, total nitrogen was analysed using combustion by a LECO Trumac nitrogen analyser. A standard curve made of EDTA was used for quantification. A nitrogen-to-protein conversion factor of 5 [173] was used.

The Dumas method is the fastest protein determination method and was therefore used when analysing protein of all seaweed samples in study I. It was also used for comparison with the amino acid data in study III, which allowed us to compile our own nitrogen-to-protein conversion factors for the three species. Those factors were not used further, but were an attempt to investigate how well the suggested universal conversion factor of 5 for seaweed [173] corresponds to the particular species used in this work. The Dumas method was also used in studies II and IV, where analyses were done on the initial seaweed biomass as well as on the protein extract. To be able to compare the total protein content between the biomasses used in studies I-V, the Dumas method was also applied to the biomass in study V.

During digestion using  $\text{H}_2\text{SO}_4$ , according to Kjeldahl [216], the organic nitrogen in a sample is converted into ammonium sulphate [220]. In this thesis, the ammonium content was thereafter analysed using HPIC, in contrast to the original version in which titration is used. Differently from the Dumas method, inorganic nitrogen will not be detected since it is incompletely converted to ammonium [223]. A standard curve made of  $(\text{NH}_4)_2\text{SO}_4$  was used for quantification and, as for the Dumas method, a nitrogen-to-protein conversion factor of 5 [173] was used. Total nitrogen using Kjeldahl was determined for selected samples emerging in study III in order to compare with the results obtained using Dumas.

#### 4.8.2 Estimation of protein nutritional quality

A way to estimate protein quality is to calculate the ‘amino acid chemical score’. This score is calculated based on a reference protein, e.g. egg [225], or based on the human requirements of amino acids [226]. The amino acid score of dried seaweeds and achieved protein extracts thereof in study III was calculated based on the requirements of amino acids for adults stated by FAO/WHO/UNU [71]. The score is determined by the limited essential amino acid in respective sample and was calculated according to **Equation 10**.

$$\text{Equation 10: Amino acid chemical score} = \left( \frac{\text{mg EAA/ g seaweed protein}}{\text{amino acid requirement in mg EAA/ g protein}} \right) * 100$$

#### 4.8.3 Fatty acids (studies I and III)

Fatty acids of dried seaweeds and extracts were directly trans-esterified and analysed using GC-MS, as earlier described in section 4.5.2. Fatty acids were analysed in all samples achieved from the different cultivation conditions in study I, to evaluate the effect of the different cultivation conditions. Since residuals of fatty acids, especially the LC-PUFA, could give an added value to the seaweed protein extracts, fatty acids were also analysed in both crude seaweeds and the obtained protein extracts in study III.

#### 4.8.4 Ash (studies I-IV)

Seaweed is known to contain high levels of minerals, which could be positive from a nutritional point of view, a salt-replacer perspective and from the perspective of enhancing the umami-taste arising from glutamic acid [56]. To evaluate how the different post-harvest treatments done prior to drying influenced the mineral content, ash was analysed in all seaweed biomasses of study II. To know where the minerals end up during the protein recovery processes, ash was also analysed in crude seaweeds and in extracts in study III. In studies II-III, the ash content was determined by combustion at 550 °C. Ash was also analysed in the different fractions obtained during sequential recovery of macromolecules in study IV. However, due to limited sample amounts, in this study, it was instead analysed using thermogravimetric analysis (TGA). TGA was also applied to the samples in study I to evaluate the effect of the different cultivation conditions on the ash content.

#### 4.8.5 Methods for evaluating the polysaccharide extracts (study IV)

For the polysaccharide fractions extracted in study IV, no quantitative analyses were carried out; instead, qualitative information was achieved using Fourier Transform Infrared (FTIR) Spectroscopy, Nuclear Magnetic Resonance (<sup>1</sup>H NMR), Size-Exclusion Chromatography (SEC) and High Performance Anion Exchange Chromatography with pulsed Amperometric Detection (HPAEC-PAD).

To investigate the potential commercial use of the carrageenan-enriched fraction, its gel-forming properties were investigated after addition of both K<sup>+</sup> and Ca<sup>2+</sup>. Carrageenan is a group of linear sulphated dimers of galactan (β-D-galactose and α-D-galactose) [35, 101]. Depending on the level of sulphatation, carrageenan is divided into κ-, ι-, and λ-carrageenan [227]. κ-carrageenan has one sulphate group per dimer, ι-carrageenan two and λ-carrageenan three [35, 101, 227]. κ-carrageenan forms a gel in the presences of K<sup>+</sup>, and ι-carrageenan in the presence of Ca<sup>2+</sup>, whereas λ-carrageenan does not form a gel at all [101]. Hence, based on the gels formed with K<sup>+</sup> and Ca<sup>2+</sup>, respectively, it is possible to determine whether κ or ι lambda carrageenan dominated in the extracted fraction.

## **4.9 Statistical analysis**

Data are presented as mean values. Error bars show standard deviations (stdv) when  $n > 2$  and  $(\text{max value} - \text{minimum value})/2$  when  $n=2$ . Statistical analyses were performed using SPSS (IBM Corp., Armonk, NY, USA), R [228] or JMP 13. To determine if there were significant differences between  $>2$  data groups, ANOVA was performed. t-test was used when only two groups were to be compared. Differences were denoted as significant when  $p \leq 0.05$ . More specific information can be found in the respective papers I-V.

## 5. RESULTS AND DISCUSSION

### 5.1 Composition of seaweed biomasses (*P. umbilicalis*, *U. lactuca* and *S. latissima*) and effects of cultivation conditions, pretreatments and drying

The composition of the seaweed biomass has a great impact on its potential as a future food. In the following section, the composition of the used biomasses and effects of cultivation condition, pretreatments and drying technique are presented.

#### 5.1.1 Protein

The protein content in the biomasses used in this work varied between species, but also within species. **Table 2** shows the total protein content obtained based on nitrogen analyses using a nitrogen-to-protein conversion factor of 5 [173] for the seaweeds used in studies I-V. Data for study III correlated well with total amino acids, see **Table 11**. Note that same biomass was used in studies III and IV.

**Table 2:** Protein content based on total nitrogen analyses (using a nitrogen-to-protein conversion factor of 5 [173]), in *Ulva lactuca*, *Porphyra umbilicalis* and *Saccharina latissima* used in studies I-V. x denotes that the species was not included in the study. Data are shown as mean values  $\pm$  (max-min)/2, (n=2).

	Total protein (% dw)		
	<i>U. lactuca</i>	<i>P. umbilicalis</i>	<i>S. latissima</i>
Study I	5.3 $\pm$ 0.1-17.6 $\pm$ 0.4	x	x
Study II	7.3 $\pm$ 0.3-10.3 $\pm$ 0.2	13.6 $\pm$ 0.3-20.3 $\pm$ 0.2	x
Study III	18.5 $\pm$ 0.0	30.2 $\pm$ 0.1*	10.0 $\pm$ 0.1
Study IV	x	30.2 $\pm$ 0.1*	x
Study V	17.0 $\pm$ 0.04	x	x

\*The same biomass was used in studies III and IV

#### Effects of different cultivation conditions (study I)

As can be seen in **Table 3**, the highest protein content in study I (13.8-17.6%) was, as expected, achieved by the addition of nitrate (150 and 500  $\mu$ M) during the cultivation. This is in accordance with what earlier has been seen for *U. rigida* [180, 181]. The combination with ambient phosphorous levels resulted in the highest protein content, 17.6%. The protein content was also favoured by low light (50  $\mu$ mol photons  $\text{m}^{-2}\text{s}^{-1}$ ) and temperature (13  $^{\circ}\text{C}$ ). Under high light (160  $\mu$ mol photons  $\text{m}^{-2}\text{s}^{-1}$ ) and high temperature (18  $^{\circ}\text{C}$ ), the opposite was seen, in accordance with what earlier was found for *U. lactuca* [182, 193]. Contradictory to this, several studies on *Ulva* spp. have, however, shown increased protein content with increased temperature [180, 181, 183]. The pH had no effect on the protein content. This is similar to what Liu and Zou [182] found for *U. lactuca*, the content of soluble proteins (in phosphate buffer) were unaffected by pH. Opposite to this, Chen et al. [196] found decreased content of soluble proteins in *U. lactuca* with decreased pH.

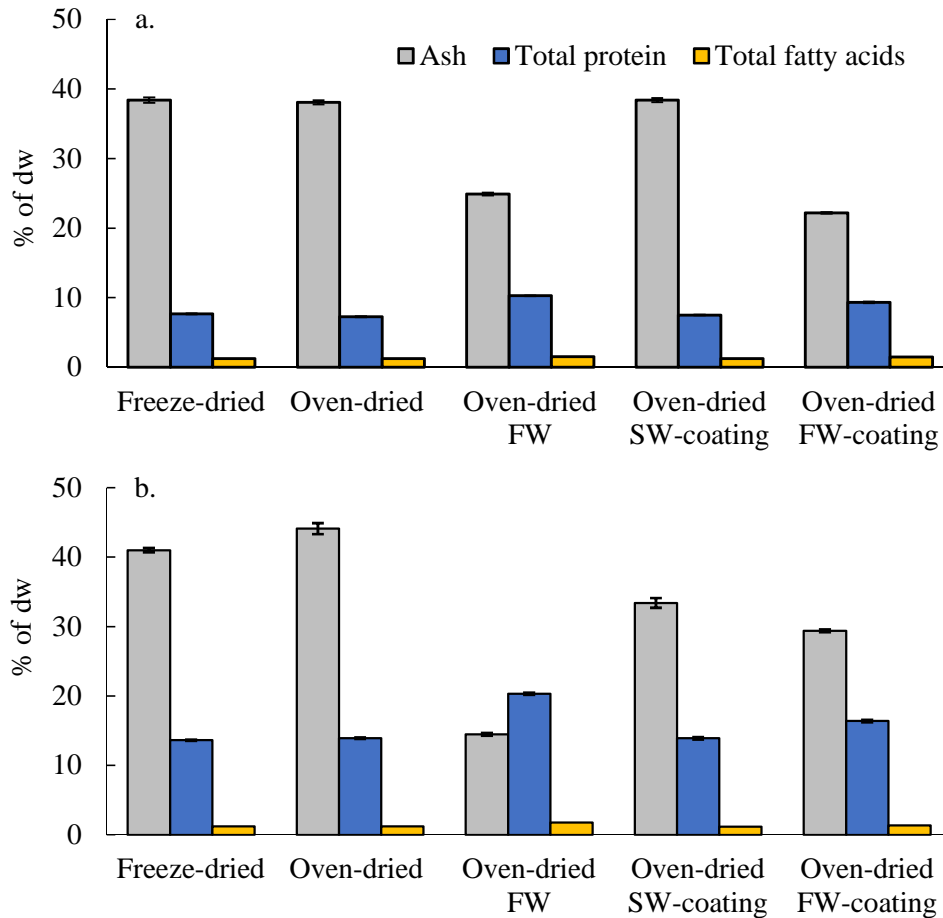


**Table 3:** Protein content in *Ulva lactuca* biomass obtained during alternative cultivation in study I. *H* denotes the highest level used, *M* the medium level used, *L* the lowest level used and *A* the ambient level for each varied parameter, see section 4.3. Data are shown as mean values  $\pm$  stdv, ( $n=4-6$  for light and temperature experiments,  $n=8$  for pH experiments and  $n=4$  for nutrient experiments).

Experiment	Light/Temperature					
Levels of light and temperature	H/H	H/L	L/H	L/L	M/H	M/L
Protein (% dw)	5.9 $\pm$ 0.5	7.8 $\pm$ 1.6	8.1 $\pm$ 1.6	10.7 $\pm$ 2.2	6.9 $\pm$ 0.7	9.1 $\pm$ 1.1
Experiment	pCO <sub>2</sub>					
Levels of pCO <sub>2</sub>	L	A	H			
Protein (% dw)	10.7 $\pm$ 1.2	11.9 $\pm$ 1.2	11.4 $\pm$ 0.9			
Experiment	Nitrate/Phosphate					
Levels of Nitrate/Phosphate	A/A	M/H	H/A	H/H	M/A	A/H
Protein (% dw)	5.1 $\pm$ 0.2	13.8 $\pm$ 1.2	17.6 $\pm$ 0.4	16.2 $\pm$ 0.8	16.2 $\pm$ 1.1	5.3 $\pm$ 0.1

#### *Effects of pretreatments and drying techniques (study II)*

The wash-out of minerals during soaking of the seaweed in freshwater changed the mass balance within the crude composition and explains the variation in protein content of study II, **Figure 10**. Soaking in freshwater could hence be a quick alternative to increase the protein content in seaweed by the partial removal of minerals and potential other non-protein compounds. However, care must be taken so that no key nutrients such as water-soluble vitamins are lost in such a treatment. The drying type (freeze-drying or oven-drying) had no effect on the total protein content of untreated *U. lactuca* and *P. umbilicalis*, in accordance with what has earlier been seen for *Sargassum hemiphyllum*, *Sargassum hemislowianum*, *Sargassum patens* and *S. latissima* [119, 122, 124, 229]. In those studies, drying was performed at 60 °C [119, 122, 229] and 40 °C [124]. For *Ulva* spp., higher protein content was found in oven-dried samples (70 °C) compared to freeze-dried ones, which is explained by the authors by the longer drying time used for the freeze-dried sample [123]. However, both in study II and the other above-mentioned studies [119, 122, 124, 229], the freeze-drying required a longer time than the oven-drying, and there this effect was not seen. Hence, no clear pattern is seen in the literature on how the protein content in seaweed varies with different drying techniques. Differences between species and specific drying temperature used is most likely contributing to the discrepancies.



**Figure 10:** Ash, total protein and total fatty acid content of freeze-dried, oven-dried, oven-dried-FW, oven-dried SW-coated and oven-dried FW-coated samples of a) *Ulva lactuca* and b) *Porphyra umbilicalis* prior to storage in study II. FW=soaked in freshwater, SW-coating=soaked in saltwater with whey coating, FW-coating=soaked in freshwater with whey coating. Data are shown as mean values  $\pm$  (max-min)/2, (n=2) for ash and total protein. Analysis of fatty acids was performed on single samples.

#### Variation in protein content between species and studies (studies I-V)

*P. umbilicalis* had the highest protein content in both studies II and III (13.6-20.3% in study II and 30.2% in study III) compared to *U. lactuca* (7.3-10.3% protein in study II and 18.5% in study III). *S. latissima* was only included in study III and had the lowest protein content, 10.0%. The order of protein content between the species was in line with earlier studies, which shows that red and green species have more protein than brown seaweed species [53, 54, 66-70].

In studies II and III, the cultivation conditions were the same, but the seasons differed (autumn for study II and winter for study III). The variation in protein content for *P. umbilicalis* and *U. lactuca* between studies II and III (13.6% and 30.2% vs. 7.7% and 19.2%, respectively) is in accordance with what others have seen, that the protein content of seaweed varies over the season [69, 78, 79, 85, 86]. No clear pattern on when the reported protein content is the highest is seen in those studies. For *U. lactuca*, the protein content in study III was even higher than in study I (5.3-17.6%), where alternative cultivation was performed. Normally, the nutrient levels of the seawater are highest during winter time, why one can speculate that this explains the high protein content received in study III compared to in study II. The protein content in *U. lactuca*

used in study V was 17% and thus ranked it in between *U. lactuca* used in studies I-III. This biomass was cultivated during spring.

For *S. latissima*, the protein content found in study III (10.0%) is in the higher range of what others have reported for this species, ~3-11% [52, 69, 85, 86]. For *U. lactuca*, most of the protein content found corresponds to what others have reported, ~8-27% [54, 67, 70, 74, 79, 84], however, the lowest protein content we found in study I (5.1-7.8%) are slightly below earlier reported data. The protein content in *P. umbilicalis* are not as well studied as for *U. lactuca* and *S. latissima*. However, the protein content found for *P. umbilicalis* in studies II and III is similar to the ones reported for other *Porphyra* spp., ~17-25% [53, 81, 91]. It should be stressed that the relatively high protein content found for *P. umbilicalis* in study III, 30.2%, is close to the content found in soybeans, ~35–45% dw [230-232]. This makes *P. umbilicalis* a promising alternative to soy, which is currently the most used crop for producing vegetarian protein isolates.

#### *Essential amino acids in P. umbilicalis, U. lactuca and S. latissimia (study III)*

The content of essential amino acids in crude *P. umbilicalis*, *U. lactuca* and *S. latissimia* of study III were 34.7%, 35.0% and 38.1% of the total amino acids, respectively. This corresponds to 11.0, 6.8 and 3.8% essential amino acids in the three species on a dw basis, which is lower than in soy (~15-20%) [230, 231]. Still, several of the essential amino acids found in the three seaweed species fulfil the recommended values for adults, see **Table 4**. For instance, *U. lactuca* and *S. latissima* fulfil the requirements of methionine and cysteine, which often are limited in legumes [233] and *P. umbilicalis* fulfil the requirement of lysine, which often is limited in cereals [234, 235]. This confirms the hypothesis that seaweed is a promising source for food protein ingredients. It should be noted that tyrosine was by mistake counted as an essential amino acid in study III and therefore slightly higher EAA-values were reported in paper III.

**Table 4:** Essential amino acids in *Porphyra umbilicalis*, *Ulva lactuca* and *Saccharina latissima* used in study III and the recommended values according to FAO/WHO/UNU [71]. Data are shown as mean values  $\pm$  stdv, (n=3).

Essential amino acids (mg/g protein)	<i>P. umbilicalis</i>	<i>U. lactuca</i>	<i>S. latissima</i>	Recommendation [71]*
Histidine	4.9 $\pm$ 0.6	3.2 $\pm$ 0.6	23.8 $\pm$ 0.9	15
Isoleucine	33.8 $\pm$ 1.2	42.6 $\pm$ 0.5	50.6 $\pm$ 1.9	30
Leucine	66.2 $\pm$ 1.1	72.1 $\pm$ 0.8	75.8 $\pm$ 2.9	59
Lysine	72.9 $\pm$ 2.4	32.8 $\pm$ 5.0	40.1 $\pm$ 1.0	45
Methionine+	17.1	36.5	45.9	22
Cysteine	(2.8 $\pm$ 0.9)+(14.3 $\pm$ 1.8)	(11.7 $\pm$ 0.2)+(24.8 $\pm$ 2.4)	(12.7 $\pm$ 0.6)+(33.2 $\pm$ 1.9)	
Phenylalanine+	79.3	85.8	87.0	30
Tyrosine	(41.6 $\pm$ 2.3+37.7 $\pm$ 1.4)	(48.8 $\pm$ 0.7+37.0 $\pm$ 2.3)	(49.9 $\pm$ 0.7+37.1 $\pm$ 1.4)	
Threonine	53.2 $\pm$ 1.3	51.7 $\pm$ 1.2	37.7 $\pm$ 0.9	23
Valine	57.4 $\pm$ 0.6	60.8 $\pm$ 0.7	55.9 $\pm$ 1.0	39
Tryptophan	na	na	na	6

\*Based on the recommended intake of 0.66 g protein/kg per day.

na=not analysed

### 5.1.2 Fatty acids

#### *Effects of different cultivation conditions (study I)*

The total fatty acid content in study I was positively affected by low light ( $50 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ ), high temperature ( $18^\circ\text{C}$ ) and addition of nitrate ( $150$  and  $500 \mu\text{M}$ ). This is in accordance with the findings of Goa et al. [180] that higher temperature ( $18^\circ\text{C}$  compared to  $14^\circ\text{C}$ ) and higher nitrate levels ( $150 \mu\text{M}$  compared to  $6 \mu\text{M}$ ) increased the lipid content in *U. rigida*.

#### *Effects of pretreatments and drying techniques (study II)*

As described for the proteins, the washout of minerals during soaking in freshwater in study II, resulted in slightly higher fatty acids content in soaked samples compared to the un-soaked ones, see **Figure 10**. No effect of drying method was seen on the total fatty acid content in study II, which is in accordance with what earlier has been seen for *Ulva* sp. [123]. For *S. hemiphyllum*, higher fatty acid content was recorded in freeze-dried samples than in oven-dried samples [229]. The higher drying temperature ( $60^\circ\text{C}$ ) used in that study compared to in study II ( $40^\circ\text{C}$ ) could have induced lipid oxidation and thus loss of fatty acids.

#### *Variation in fatty acid content between species and studies (studies I-III)*

The total fatty acid content in the crude seaweeds in studies I-III was low, 1.1-2.5% of the dw, (**Table 5-7**), which is in agreement with what has been reported by others [20, 66, 70, 74, 85]. However, the relative amount of PUFAs was high, 31.9-73.9% of total fatty acids, which still makes the lipid pool interesting from a nutritional perspective.

In studies II and III, the n-6/n-3 ratio was  $< 1$  for all crude seaweeds (0.27-0.87), which, as mentioned earlier, is considered good since a diet with a low n-6/n-3 is associated with a reduced risk for cardiovascular and inflammatory diseases [99]. In study I, the n-6/n-3 ratio varied from 0.39 to 1.61, depending on the cultivation conditions. Lowest n-6/n-3 ratios (0.40-0.44) were obtained by nitrate addition, **Table 5**, which corresponded well with those samples having the highest ALA and docosapentaenoic acid (DPA) levels.

In *P. umbilicalis*, the content of the LC n-3 PUFA EPA was especially high, exerting 32-44% of the total fatty acids in studies II and III. In *U. lactuca*, no or low levels of EPA were found, although it contained a substantial amount of ALA (11-25% of total fatty acids), a precursor to the LC n-3 PUFAs. Also, low levels of DPA were found in *U. lactuca*, while this fatty acid was absent in *P. umbilicalis* and *S. latissima*. DPA has earlier been detected in *U. lactuca* by both Maehre et al. [70] and Ortiz et al. [74], but similar to us, these studies did not report on DPA in brown [70, 74] or red seaweed [70]. In *S. latissima*, both ALA and EPA were found. They constituted 8.5 and 18.5% of total fatty acids, respectively. Our results are in accordance with other studies, reporting that red seaweed contain more EPA than both green and brown seaweed [20, 53, 66, 70, 97]. Veide Vilg et al. [85] reported that the LC n-3 PUFA DHA was recorded in wild *S. latissima* harvested at the Swedish west coast in August. However, no DHA was found in *S. latissima* in June in the same study [85]. No DHA was found in the semi-cultivated *S. latissima* used in study III, which was cultivated during winter time.

**Table 5:** Total and unsaturated fatty acids as well as n-6/n-3 ratios in *Ulva lactuca* biomass obtained during alternative cultivation in study I. *H* denotes the highest level used, *M* the medium level used, *L* the lowest level used and *A* the ambient level for each varied parameter, see section 4.3. Data are shown as mean values  $\pm$  stdv, (*n*=4-6 for light and temperature experiments, *n*=8 for pH experiments and *n*=4 for nutrient experiments).

	Light/temp			pH			Nitrate/Phosphorous								
	H/H	H/L	M/H	M/L	L/H	L/L	L	A	H	H/H	H/A	M/H	M/A	A/H	A/A
Total FA (% dw)	1.4±0.1	1.1±0	1.4±0.1	1.2±0.1	1.6±0.2	1.3±0.1	1.8±0.1	1.9±0.1	1.8±0.1	1.9±0	1.7±0	1.8±0.1	1.6±0.1	1.3±0	1.3±0
Unsaturated FA (% of total FA)															
MUFA	18.8±0.7	19.4±0.7	18.1±0.4	18.4±0.4	17.5±1.5	17.8±0.7	12.8±0.5	11.9±0.3	12.1±0.3	11.5±0.3	14.3±0.4	11.9±0.3	14.3±0.7	14.6±0.2	14.7±0.3
PUFA	34.3±1.2	31.9±1.1	36.1±2.2	35.7±1	38.3±3	36.4±1.8	48.9±0.7	49.8±0.5	49.8±0.3	47.6±0.4	45.2±0.1	47±0.6	44.8±0.3	45.4±0.4	45±0.4
PUFA n-3	13.9±0.8	18±0.9	15.8±1.5	21.1±1	17.6±2.9	21.7±1.6	28±0.5	30.2±0.4	29.8±0.6	33.9±0.2	32.1±0.2	33.3±0.2	31.2±0.6	28.3±0.6	28.5±0.7
PUFA n-6	20.4±0.6	13.9±0.3	20.3±0.9	14.6±0.7	20.7±0.9	14.7±0.6	21±0.6	19.6±0.7	20±0.9	13.7±0.2	13.1±0.2	13.7±0.6	13.6±0.5	17±0.3	16.6±0.3
n-6/n-3	1.47±0.08	0.77±0.03	1.29±0.10	0.69±0.05	1.2±0.19	0.68±0.06	0.75±0.03	0.65±0.03	0.67±0.04	0.40±0.01	0.41±0.01	0.41±0.02	0.44±0.02	0.60±0.02	0.58±0.02

**Table 6:** Total and unsaturated fatty acids as well as n-6/n-3 ratios in *Porphyrha umbilicalis* and *Ulva lactuca* directly after drying in study II. FW=soaked in freshwater, SW-coating=soaked in saltwater with whey coating, FW-coating=soaked in freshwater with whey coating.

	<i>P. umbilicalis</i>				<i>U. lactuca</i>			
	Freeze-dried	Oven-dried	Oven-dried-FW	Oven-dried-SW	Freeze-dried	Oven-dried	Oven-dried-FW	Oven-dried-SW
Total FA (% dw)	1.2	1.2	1.8	1.2	1.2	1.3	1.5	1.3
Unsaturated FA (% of total FA)								
MUFA	11.0	11.7	12.1	12.4	15.3	16.8	15.5	15.2
PUFA	62.8	61.8	62.3	60.5	57.4	54.7	57.6	58.0
PUFA n-3	36.2	37.5	37.0	34.0	33.4	30.4	30.7	31.7
PUFA n-6	26.6	24.3	25.3	26.4	24.0	24.3	26.9	26.3
n-6/n-3	0.74	0.65	0.68	0.78	0.72	0.80	0.88	0.83

**Table 7:** Total and unsaturated fatty acids as well as n-6/n-3 ratios in *Porphyra umbilicalis*, *Ulva lactuca* and *Saccharina latissima* and extracts thereof in study III. Data are shown as mean values  $\pm$  stdv, (n=3 if not other stated).

	<i>P. umbilicalis</i>				<i>U. lactuca</i>				<i>S. latissima</i>			
	Dry	Traditional	pH-shift	ASE	Dry	Traditional	pH-shift	ASE	Dry	Traditional	pH-shift	ASE
Total FA (% dw)	2.5±0*	0.5±0.2	2.2±0.1	0.7±0*	2.3±0	0.7±0	5.0±0.4	1.8±0*	2.1±0.4	0.1±0	3.3±0.2	1.6±0#
Unsaturated FA (% of total FA)												
MUFA	2.6±0	9.4±0.2	5.2±1.1	2±1	11.4±0.1	16.5±0.1	13±0.1	4.4±0.2	2.4±0.2	2.2±2.2	5.4±0.4	4.5±0
PUFA	73.9±0	40.4±2.6	60.2±3.1	44.2±1.7	68.8±1	53.8±1.3	63.7±0.7	51.1±0	69.6±3.2	36.8±5.2	35.6±2.6	9.3±0
PUFA n-3	46.7±0	22.1±1.6	39.1±1.8	24.8±0.9	54.4±0.5	41.5±0.9	50.3±0.6	39.6±0	47.1±2.3	17.5±0.7	16.3±1.3	5.2±0
PUFA n-6	27.2±0.1	18.3±1	21.1±1.4	19.3±0.8	14.5±0.5	12.2±0.4	13.5±0.2	11.4±0	22.5±0.9	19.3±4.5	19.3±1.3	4.1±0
n-6/n-3	0.58±0.0	0.83±0.02	0.54±0.01	0.78±0.0	0.27±0.01	0.29±0.0	0.27±0.0	0.29±0.0	0.48±0.0	1.1±0.22	1.18±0.02	0.78±0

\* Analysis was performed in duplicate. Data are shown as mean values  $\pm$  (max-min)/2.

<sup>#</sup> Analysis was performed on single samples.

### 5.1.3 Ash

#### *Effects of pretreatments and drying techniques (study II)*

As mentioned above, study II revealed that there was a significant washout of minerals during soaking in freshwater, see **Figure 10**. The ash content in the soaked samples was therefore significantly lower than in corresponding non-soaked samples.

#### *Variation in ash content between species and studies (studies I-IV)*

The ash content in study II for both non-soaked and uncoated *P. umbilicalis* (41-44 %) and *U. lactuca* (38%) was higher than in study III (23% and 26%, respectively) and for *U. lactuca*, also than in study I (~20%). For *S. latissima*, the ash content in study III was as high as 50%. It is well known that seaweed can have very high ash content [79, 80, 90] since it contains a variety of minerals [90, 109, 113, 236]. It has also been shown that the ash content can vary e.g. with season [78, 79, 85, 86].

When reanalysing the *P. umbilicalis* biomass from study III using TGA combustion in study IV, the achieved ash content was lower (9.1% compared to 23%). TGA combustion is done at 800 °C, while in study III, combustion was done at 550 °C. Also, during TGA combustion, the char content is determined at 800 °C under N<sub>2</sub>-atmosphere prior to the determination of ash. At this stage, polysaccharides depolymerise into monosaccharides, which in turn decompose into carbon and water. Protein decompose into amino acids and then further to ammonium and carbon dioxide. Possibly, a temperature of 550 °C is not enough to fully combust the char of the sample, why some char could end up in the ash-fraction. Another reason for the differences in ash content for *P. umbilicalis* between studies IV and III could be that the composition of seaweed differs within the thallus. In study IV, a very low sample quantity (4mg) was used in TGA-analysis, providing uncertainty in how representative such a small sample is.

## 5.2 Influence of pretreatments, drying techniques and storage conditions on the quality of *P. umbilicalis* and *U. lactuca* (study II)

### 5.2.1 Formation of lipid oxidation products

Lipid oxidation could affect both the nutritional value and the flavour of the seaweed biomass and is therefore an important quality parameter. To the best of our knowledge, no other studies have reported any specific oxidation products in seaweed. In study II, analyses of free aldehydes in the seaweeds directly after drying revealed 1.0-4.9 µg MDA/g, 0.1-0.3 µg HHE/g and 0.02-0.2 µg HNE/g. Due to the novelty in analysing these compounds in seaweed, the figures cannot be compared with others. However, Chen et al. [196] analysed TBARS in fresh *Pyropia haitanensis* and *U. lactuca*. Achieved TBARS levels in that study [196], when recalculated based on µg/g dw, were ~ 5.4-11.9 µg MDA-equivalents/g, hence somewhat higher than the obtained MDA levels in study II. This agrees with the fact that the non-specific TBARS method includes all carbonyls, not only MDA.

When recalculating reported levels of MDA and HHE in herring and salmon (280µg HHE and 10µg MDA/100g and 16µg MDA and 0.83µg HHE/100g wet weight (ww), respectively) [237] to a dw basis, using moisture content levels reported by the Swedish Food Agency (~71% in both herring [238] and salmon [239]), it corresponds to 0.6-9.7µg MDA/g and 0.03-0.3µg HHE/g. When doing the same calculation for HHE and HNE values reported for mackerel (11µg HHE/kg ww and 7µg HNE/kg ww [240] and using a moisture content of ~55% [241]), it corresponds to 0.024µg HHE/g and 0.0156µg HNE/g. For fresh walnuts and peanuts, the

reported MDA levels are 0.44 and 0.72 µg/g, respectively [242]. Hence, the initial aldehyde levels in the seaweeds lay in the upper range of the reported values in various foods.

#### *Effects of pretreatments and drying techniques*

Both for *U. lactuca* and *P. umbilicalis* the freeze-dried samples had higher initial levels of aldehydes (MDA and HHE for *U. lactuca* and HHE and HNE for *P. umbilicalis*) than the oven-dried samples, see Figure 1-3 in study II. The freeze-drying *per se* thus unexpectedly induced more lipid oxidation than oven-drying, or, the oven-drying stimulated binding of the free aldehydes to other macromolecules. MDA, HHE and HNE are all very reactive and can cross-link, e.g. to proteins [243-245], something that could be enhanced during the elevated oven-drying temperatures (40 °C). Soaking in freshwater had no effect on the initial levels of aldehydes. For *U. lactuca* the coated samples had lower initial levels of aldehydes compared to uncoated samples. This was, however, not seen for *P. umbilicalis*. Generally, the magnitude of HHE reached in the samples was higher than for HNE, which is in accordance with the higher levels of n-3 fatty acids compared to n-6 fatty acids in the seaweeds, **Table 6**.

#### *Effects of storage conditions*

Throughout the whole storage, the formation of MDA, HHE and HNE was moderate for all samples. Nevertheless, there are some interesting trends worth mentioning. Light had a larger impact on the aldehyde development in *U. lactuca* than in *P. umbilicalis*, which could be due to a higher chlorophyll content in green compared to red seaweeds [246, 247]. The kinetics for HHE and HNE development, Figure 2-3 study II, (especially for light and semi-light stored *U. lactuca*) were similar, with a lag phase between day 0-150, a log phase between day 150 and day 230 or 300, and thereafter a more stationary phase. This pattern is typical for the lipid oxidation reaction and has been seen in many other studies, e.g. of fish muscle [248-250]. For *U. lactuca*, the start of the log phase corresponded to the time point where the ascorbic acid loss also accelerated, i.e. > day 150. Which is the hen and the egg here is difficult to pinpoint; either the accelerated lipid oxidation induced co-oxidation of the ascorbic acid or the levels of ascorbic acid had decreased to a level where it no longer could provide any antioxidative capacities. Despite the moderate development of MDA, HHE and HNE during storage, their high reactivity [136, 243-245] could have affected the seaweed protein quality, something that is still to be explored.

### **5.2.2 Fatty acid degradation**

Along with lipid oxidation, fatty acids are destroyed, something that reduces the nutritional value of the biomass. The change in fatty acid content by the different pretreatments and drying techniques as well as after one year of storage was therefore considered an important quality parameter.

#### *Effects of pretreatments and drying techniques*

As mentioned above (see 5.1.2), the major effect of the different pretreatments was that the washout of ash during soaking in freshwater resulted in higher fatty acid levels in those samples, see **Figure 10**. The drying technique did not affect the fatty acid content.

#### *Effects of storage conditions*

There was a great loss of fatty acids during the storage, which was more pronounced for unsaturated fatty acids and for the samples stored in light. In the latter samples, 53.0-98.6% of all PUFAs degraded, the exact amount depending on species and sample type. However, also during storage in darkness, the PUFA were not fully retained, and the loss there varied between



20.3-80.1%, which is accordance with what Schmid et al. [132] showed for *L. digitata* and *P. palamata*. In these species, the PUFA content was less than half of the initial value after 22 months of storage in darkness at RT. The difference between samples stored light and dark in study II was more pronounced regarding their loss of PUFA than their obtained levels of oxidation products. Thus, it is likely that lipid oxidation of the dried seaweeds primarily yielded other lipid oxidation products than free MDA, HHE and HNE. Examples are hexanal, pentanal, nonanal, butanal and pentenol, which are common lipid oxidation markers for muscle tissues and vegetable food sources [251, 252]. The greatest difference in loss for PUFA between dark and light storage (3.3 times) was seen in freeze-dried *U. lactuca*. For freeze-dried *P. umbilicalis*, the difference was less (1.2 times). This is due to that the dark-stored *P. umbilicalis* sample lost 80.1% of the initial PUFA content, which can be compared to 29.5% for *U. lactuca* stored dark. Generally, the *P. umbilicalis* samples stored both dark and semi-light lost more PUFA than the *U. lactuca* samples stored under the same conditions. Thus, the specific light condition used had a larger impact on the loss of PUFA for *U. lactuca* than for *P. umbilicalis*. Green seaweed generally contains more chlorophyll than red seaweed [246, 247]. Even if chlorophyll acts as a photosensitiser in light [126], it has also been shown to work as an antioxidant in darkness [253, 254], and hence could explain why *U. lactuca* lost less PUFAs than *P. umbilicalis* when stored dark. For *U. lactuca*, the coated samples lost less PUFAs compared to uncoated samples, and hence the coating seemed to protect the PUFAs in *U. lactuca* during storage. For *P. umbilicalis*, the coated samples were stored only in semi-light, and no protective effect was seen compared to the un-coted samples stored under the same conditions.

### 5.2.3 Ascorbic acid

Ascorbic acid is often used as a quality indicator of food since it is very sensitive and can easily be degraded [137]. Since it can work both as an antioxidant and a pro-oxidant during lipid oxidation, it is an interesting marker to follow along with this.

#### *Effects of pretreatments and drying techniques*

The soaking of *U. lactuca* and *P. umbilicalis* in freshwater concentrated the ascorbic acid levels, just as it concentrated proteins and fatty acids, **Table 8**. This indicates that the soaking acted only on the surface and did not wash away intracellular water-soluble components. For *P. umbilicalis*, the choice of drying method did not affect the ascorbic acid level, contrary to what has been seen for *S. hemiphyllum*, where freeze-dried biomass contained significantly more ascorbic acid than the oven-dried counterpart [229]. For *U. lactuca*, the oven-dried control had a lower ascorbic acid value (335 µg/g) directly after drying compared to the freeze-dried sample (547 µg/g). However, due to the similar levels seen after 90 days of storage (552 µg/g and 570 µg/g, respectively), it is hypothesised that this rather is a result of better extractability from the freeze-dried than oven-dried sample at that point. Hamid et al. [197] found higher extractability of metabolites from freeze-dried than oven-dried biomass for *Cladosiphon okamuranus*, *Saccharina japonica* and *Undaria pinnatifida*.

The initial ascorbic acid levels were significantly lower in freshwater coated *U. lactuca* and in saltwater coated and freshwater coated *P. umbilicalis* compared to the respective uncoated samples, see **Table 8**. The pH of the coating solution made from freshwater was lower (4.55) than the ambient pH of the seawater in study I (8.1) and lower than the pH of the *U. lactuca* and *P. umbilicalis* used in study III, 4.8 and 6.1, respectively. Ascorbic acid is sensitive to pH changes, and this could explain the lower initial levels in the coated samples. Ascorbic acid is known to reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  and  $\text{Cu}^{3+}$  to  $\text{Cu}^{2+}$  in the presence of oxygen and is thereby oxidised

to dehydroascorbic acid [204, 255]. This reaction has earlier been seen to be inhibited by NaCl [255, 256] since the chloride ions can interact with the iron and copper ions and thereby partly hinder them from interacting with ascorbic acid. The pH difference between the coating solution and *U. lactuca* was less than the difference between the coating solution and *P. umbilicalis*. Possibly, the effect from NaCl in the saltwater-coated *U. lactuca* dominated over the effect from the lower pH, and hence the net effect was protective. This could explain the higher initial ascorbic acid level in this sample compared to the other coated samples.

#### *Effects of storage conditions*

During all storage conditions in study II, there was a loss of ascorbic acid in all sample types. However, for *U. lactuca*, the degradation was slower up to 150 days, after which it accelerated, Figure 4 study II. During this period, there was a slight protective effect from keeping the samples dark. For *P. umbilicalis*, the degradation of ascorbic acid started immediately, and there was no difference between the different light conditions. Ascorbic acid is very sensitive to e.g. oxygen, light and temperature [137]. In study II, the samples had full access to oxygen as they were stored in Ziplock bags. Possibly this aerobic atmosphere overshadowed the effect from light. For both species, the freeze-dried samples had lost most ascorbic acid up to 230 days (90-92% for *U. lactuca*, depending on the light condition and 100% for *P. umbilicalis*, regardless of light condition). Due to the drastic decrease of ascorbic acid during the storage of dried seaweeds, it is recommended to consume or process the biomass as soon as possible after harvest.

**Table 8:** Initial ascorbic acid levels in *Ulva lactuca* and *Porphyra umbilicalis* directly after drying. Data are shown as mean values  $\pm$  (max-min)/, (n=2). FW=soaked in freshwater, SW-coating=soaked in saltwater with whey coating, FW-coating= soaked in freshwater with whey coating.

		Ascorbic acid (ug/g)
<i>U. lactuca</i>	Freeze-dried	602.3 $\pm$ 46
	Oven-dried	368.4 $\pm$ 58.3
	Oven-dried - FW	559.2 $\pm$ 6.4
	Oven-dried - SW coating	368.3 $\pm$ 18.4
	Oven-dried - FW coating	132.8 $\pm$ 13.5
<i>P. umbilicalis</i>	Freeze-dried	798.9 $\pm$ 12.1
	Oven-dried	799.3 $\pm$ 16
	Oven-dried - FW	1098.3 $\pm$ 65.1
	Oven-dried - SW coating	198.9 $\pm$ 7.6
	Oven-dried - FW coating	161.3 $\pm$ 1.1

### 5.2.4 Change in colour

#### *Effects of pretreatments and drying techniques*

The different pretreatments and drying techniques applied had no or little effect on the initial colour of the seaweeds, as can be seen in Figure 5-7 in study II. However, the freeze-dried samples were slightly lighter than the oven-dried ones, seen as higher L-values (51 vs. 45 for *U. lactuca* and 42 vs. 30 for *P. umbilicalis*).

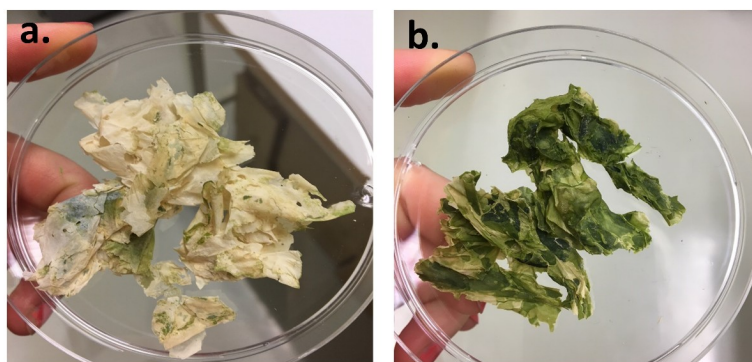
#### *Effects of storage conditions*

The trend for all samples during storage was an increase in a-value and a decrease in b-value, indicating a bleaching of the green and yellow pigments, such as chlorophyll and carotenoids. For uncoated samples, the general trend was an increase in L-value, which correlates with the changes in a- and b-value. For the coated samples, this was not as clear. From visual inspection, the coated *U. lactuca* samples appeared a bit brownish in comparison to uncoated samples, both directly after drying and during storage. This could probably be due to the Maillard reaction taking place between the reducing sugars in the whey coating and the free amino acids of the seaweed [257]. It was, however, not possible to capture this browning with the colorimetric analyses, possibly due to the high pigmentation of the seaweed which may overshadow this change.

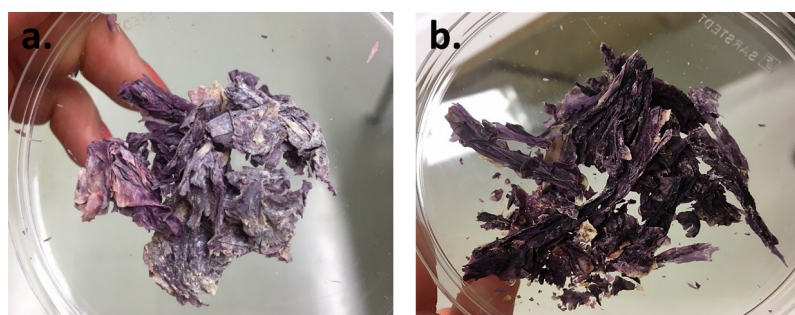
Between day 230 and day 290, the freeze-dried *U. lactuca* samples stored light got remarkably bleached when inspected visually, see **Figure 11**. This was not seen for the oven-dried sample, **Figure 12**. In accordance with this, the L-value was ~46 for the oven-dried sample and ~61 for the freeze-dried sample at day 310. Also, the total colour changes ( $\Delta E$  value) were 14.5 and 20.5 at day 310 for the oven-dried and freeze-dried samples, respectively, **Table 9**. The same tendency was seen for *P. umbilicalis*, see **Figure 13** and **Table 10**, however, it was not as clear as for *U. lactuca*.



**Figure 11:** Freeze-dried *Ulva lactuca* stored a) dark, b) semi-light and c) light for 230 days and stored d) dark, e) semi-light and f) light for 290 days.



**Figure 12:** a) Freeze-dried and b) oven-dried *Ulva lactuca* after 290 days of storage in light.



**Figure 13:** a) Freeze-dried and b) oven-dried *Porphyra umbilicalis* after 290 days of storage in light.

**Table 9:** Total colour change,  $\Delta E$ , for freeze-dried, oven-dried, oven-dried-FW, oven-dried SW-coated and oven-dried FW-coated *U. lactuca* during storage. Note that the two first values for the light storage (grey) is the same as for the dark storage since those samples were moved to light first after 90 days of dark storage. FW=soaked in freshwater, SW-coating=soaked in saltwater with whey coating, FW-coating=soaked in freshwater with whey coating.

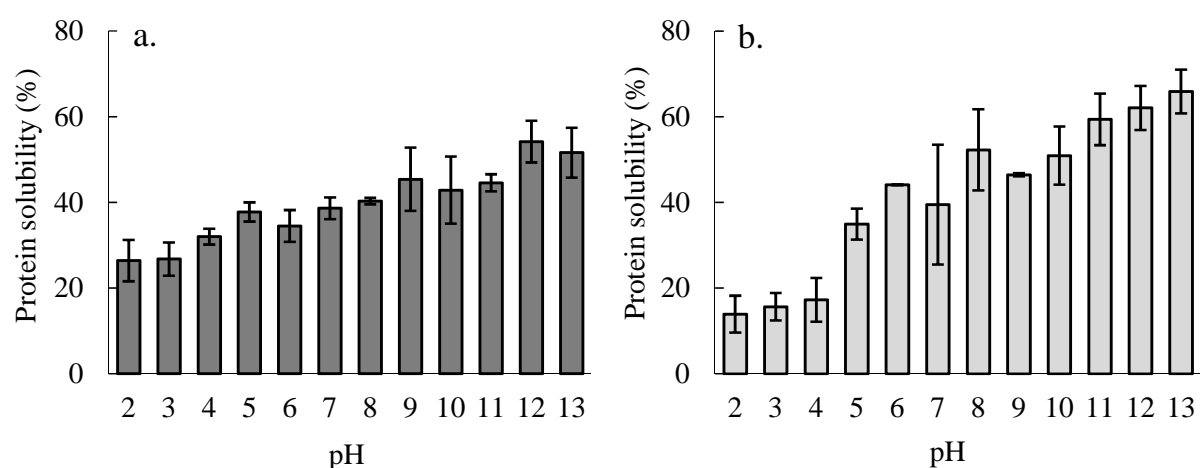
Day	Freeze-dried			Oven-dried			Oven-dried-FW			Oven-dried SW-coating			Oven-dried FW-coating		
	Dark	Semi-light	Light	Dark	Semi-light	Light	Dark	Semi-light	Light	Dark	Semi-light	Light	Dark	Semi-light	Light
30	8.2	10.7	8.2	3.9	5.4	3.9	1.6	3.0	1.6	3.7	4.9	3.7	4.5	6.1	4.5
90	11.0	10.8	11.0	2.3	5.3	2.3	3.4	1.8	3.4	3.1	5.5	3.1	2.2	3.2	2.2
150	7.2	11.7	9.5	5.0	5.9	4.2	4.6	1.2	3.1	3.4	5.8	5.6	5.4	3.5	8.7
230	5.9	8.1	6.7	4.7	2.5	3.8	8.4	3.8	8.3	9.3	5.2	4.9	4.8	5.7	6.8
310	8.9	11.5	20.5	7.2	10.5	14.5	10.8	9.1	12.2	8.8	11.8	14.1	6.7	3.2	8.2
370	10.4	15.1	23.2	12.9	14.6	15.1	8.0	13.6	14.5	19.7	18.2	18.7	17.1	13.2	17.0
520	12.3	15.2	22.6	9.0	14.8	23.5	6.8	13.9	24.0	16.3	19.9	18.1	13.0	14.0	14.4

**Table 10:** Total colour change,  $\Delta E$ , for freeze-dried, oven-dried, oven-dried-FW, oven-dried SW-coating and oven-dried FW-coating *P. umbilicalis* during storage. Note that the two first values for the light storage (grey) is the same as for the dark storage since those samples were moved to light first after 90 days of dark storage. FW=soaked in freshwater, SW-coating=soaked in saltwater with whey coating, FW-coating=soaked in freshwater with whey coating.

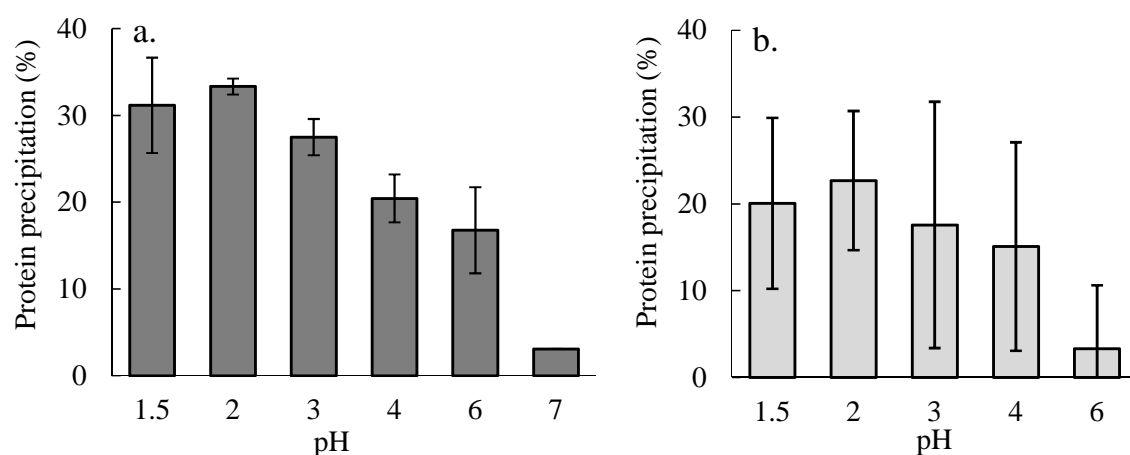
Day	Freeze-dried			Oven-dried			Oven-dried-FW			Oven-dried SW-coating			Oven-dried FW-coating		
	Dark	Semi-light	Light	Dark	Semi-light	Light	Dark	Semi-light	Light	Dark	Semi-light	Light	Dark	Semi-light	Light
30	3.2	4.4	3.2	7.0	13.6	7.0	10.5	3.2	10.5	8.5					
90	2.9	2.8	2.9	11.1	16.0	11.1	14.9	0.3	14.9	1.8					
150	7.8	8.5	10.3	13.8	4.7	9.1	2.4	2.0	0.3	2.6					
230	3.5	2.6	8.8	6.9	5.9	12.5	5.5	3.5	14.1	7.4					
370	10.2	11.7	15.8	16.5	14.7	19.7	10.1	14.4	21.2	12.4					
520		9.1			16.0			16.8		11.8					

### 5.3 Adaption and improvements of the pH-shift process to *P. umbilicalis* and *U. lactuca*

During the adaption of the pH-shift process to *P. umbilicalis* and *U. lactuca*, it was found that the protein solubility increased with increasing pH in the first step of the process, **Figure 14**, while precipitation of recovered solubilised proteins increased with decreasing pH, **Figure 15**, equal to what was found for *S. latissima* [148]. The latter is different from e.g. fish and bean proteins, which normally have high solubility at both high and low pH values [258-260]. The trend with high solubility at high pH values and low solubility at low pH values has, however, earlier been reported for microalgae (*Nannochloropsis oculata*) [168].



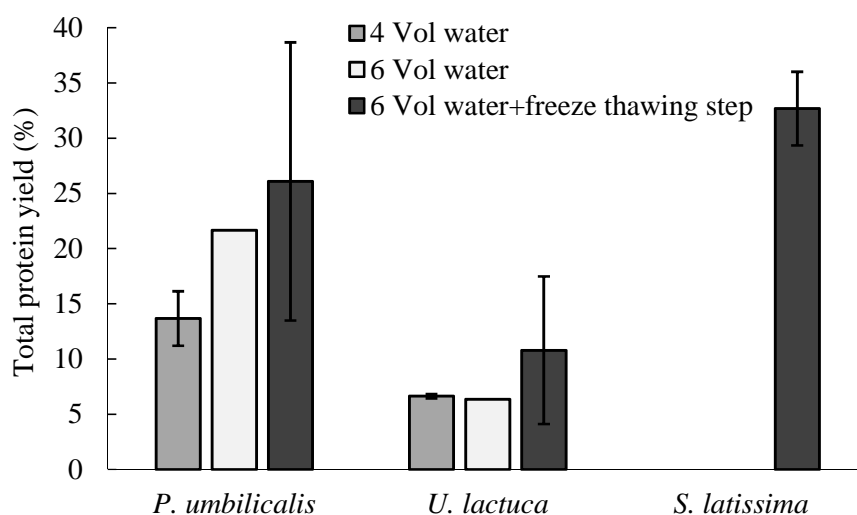
**Figure 14:** Protein solubility of a.) *Porphyra umbilicalis* and b.) *Ulva lactuca* at different pHs. Data are shown as mean values  $\pm$  (max-min)/2, (n=2).



**Figure 15:** Protein precipitation of a.) *Porphyra umbilicalis* and b.) *Ulva lactuca* at different pHs after initial protein solubilisation at pH 12 followed by centrifugation at 8000xg. Data are shown as mean values  $\pm$  (max-min)/2, (n=2).

Extraction using a larger volume of water (6 volumes as opposed to 4 volumes based on the wet weight of the seaweeds) improved the solubility and hence the total yield for *P. umbilicalis*, see **Figure 16**. This is in line with earlier results for *S. latissima* [148]. This tendency was also seen by Barbarino and Lourenco [261] for *Ulva fasciata* in an attempt to find the most efficient way to extract seaweed proteins prior to analyses with Lowry and Bradford. The lower protein recovery with a lower amount of water is partly due to the system becoming more concentrated, which will increase the loss of soluble proteins since those also are distributed in the first pellet. A larger water volume will dilute the salt provided by the seaweed and could thereby also increase the solubility of the proteins by reducing the salting-out effect. Further, as mentioned earlier, the strong gelling capacity of the polysaccharides found in red and brown seaweed reduces the extractability of seaweed proteins [150, 151]. By using a larger water volume, this gelling is reduced. The polysaccharides in *U. lactuca* do not have the same strong gelling capacity, which could explain why a larger water volume did not have the same effect on the protein solubility for this specie, see **Figure 16**.

In comparison to both fish proteins [162, 163] and microalgae proteins [168] the seaweed proteins have notably lower precipitation ability. The freeze-thawing step included before the second centrifugation enhanced the precipitation and thereby also the total protein yield for both *P. umbilicalis* and *U. lactuca*, **Figure 16**. This step was also included during pH-shift processing of *S. latissima* and increased the total protein yield from the earlier reported 16.0% [148] to 32.7%. The inclusion of a freeze-thawing step in the pH-shift process has recently again been shown to have a positive effect on the precipitation of *S. latissima* proteins by Abdollahi et al. [124]. In that study [124], freeze-thawing increased the protein precipitation from ~12 to 29% and the total protein yield from ~11 to 26%. The proteins denature when getting into contact with the interference of the unfrozen water and the ice during the freezing process [262, 263], which aids the precipitation. During freezing, the ionic strength and pH in the unfrozen liquid can change, which also affects the precipitation [262].



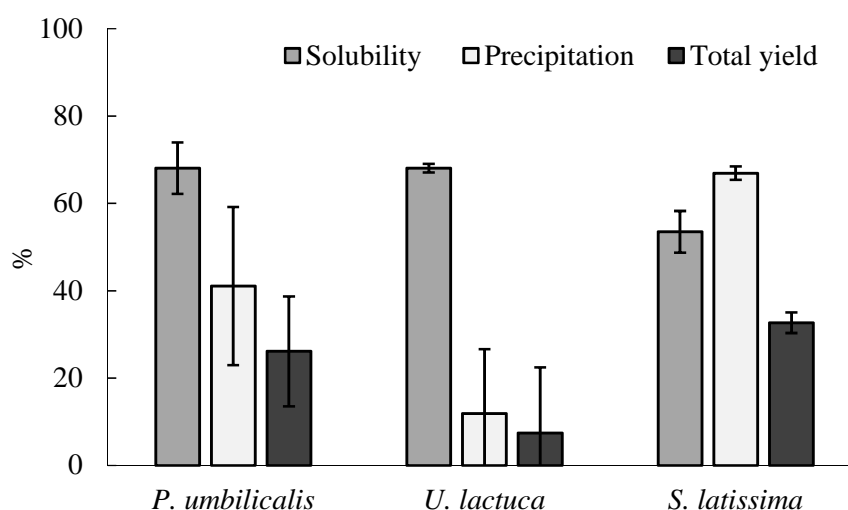
**Figure 16:** Total protein yield obtained for different variations of the pH-shift process for *Porphyra umbilicalis*, *Ulva lactuca* and *Saccharina latissima*. Data are shown as mean values  $\pm$  (max-min)/2, (n=2), except for 6 Vol water, which was only performed once. Vol=Volumes.

### 5.3.1 The final pH-shift method used (study III)

Based on the results presented above, it was chosen to use 6 volumes of water based on the wet weight of the used seaweeds for homogenisation followed by an osmotic shock. Solubility was performed at pH 12 and precipitation at pH 2. A freeze-thawing step before the second centrifugation step was also included.

During the production of seaweed protein extracts in study III, the solubility at pH 12 was equal for all three species (53-68%), **Figure 17**. The reported solubility of proteins (measured as soluble nitrogen) for *Enteromorpha sp.* at pH 12 varied between ~20-25% [159], hence lower than in study III. For *Sargassum nodosum*, alkaline extraction by incubating in 0.1 M NaOH for 1h resulted in 61% protein solubility [155]. In the same study, by replacing the incubation with 10 min sonication in 0.1 M NaOH, the solubility was increased to 73%, which indicates that sonication could be a way both to increase the solubility and reduce the process time.

The precipitation during study III was remarkably higher for *S. latissima* (67%) compared to *P. umbilicalis* (41%) and *U. lactuca* (12%), **Figure 17**. Also, the variation in the precipitation step was remarkably lower for *S. latissima*, which could be linked to the recovered protein extract being much firmer for *S. latissima* compared to the other two species. Brown seaweed could, in contrast to red and green species, contain high levels of phlorotannins [222, 264]. Phlorotannins are known to bind to, and precipitate proteins [153], and thus, could explain the higher precipitation of the proteins from *S. latissima*. However, from a protein digestibility and bioavailability perspective, the phlorotannins could be negative. The phlorotannin levels in *S. latissima* are, however, low compared to other brown species such as *Fucus vesiculosus* and *Ascophyllum nodosum* [265].



**Figure 17:** Protein solubility, precipitation and total yield during the pH-shift process of *Porphyra umbilicalis*, *Ulva lactuca* and *Saccharina latissima* using 6 volumes of water based on the wet weight of respective species, together with a freeze-thawing step before the second centrifugation. Data are shown as mean values. Results are based on analyses in homogenates and supernatants. Error bar show max-min/2 for *P. umbilicalis* and *S. latissima* (n=2) and stdv for *U. lactuca* (n=3).



## 5.4 Influence of analysis method on protein content in seaweeds and protein extracts

The protein content of seaweeds and extracts thereof were presented in study III based both on Lowry measurements and as total amino acids. A few selected samples in that study were also analysed using Dumas and Kjeldahl. The choice of protein measurement was seen to influence the obtained result, as has also been reported by others [172, 266].

For *S. latissima* the protein content achieved by Lowry measurements was higher compared to that achieved by analysing total amino acids or total nitrogen, **Table 11**. This was not seen for *P. umbilicalis* and *U. lactuca*. Since the Folin-Ciocalteu reagent used in the Lowry analysis is also used to analyse phlorotannins [221], it is likely that phlorotannins in *S. latissima* affected the results for this species. Red and green seaweed do not contain phlorotannins.

For the traditionally made protein extracts, estimation of protein based on total nitrogen content resulted in very high values, compared to the ones obtained with Lowry and total amino acid analyses, **Table 11**. These values were therefore verified by analysing the nitrogen content with a modified version of Kjeldahl. In the traditional protein extraction method, proteins were precipitated using ammonium sulphate, and the high nitrogen content indicated that the following dialysis step was insufficient. This was confirmed by analysing free nitrogen in water extracts of the traditionally made protein extracts, which revealed 10.5-16.4% free nitrogen on a dw basis. This was excessively higher than the free nitrogen of dry seaweeds, **Table 11**. When Garcia et al. [157] applied the traditional protein extraction method to *H. elongata*, they obtained 63% protein in achieved extracts based on total nitrogen analysis and only 5.4% based on analysis of total amino acids. This indicates that the dialysis step might have been insufficient in removing ammonium sulphate also in that study.

Compiling nitrogen-to-protein factors for the samples used in study III resulted in 5.3 for *P. umbilicalis*, 5.3 for *U. lactuca* and 5.1 for *S. latissima*. Those factors are close to the suggested universal seaweed conversion factor of 5 [173]. Biancarosa et al. [75] reported conversion factors for *P. umbilicalis*, *U. lactuca* and *S. latissima* harvested in Norway to be 3.92, 4.15 and 4.37, respectively, hence somewhat lower than our factors for study III. To get a better picture of the specific conversion factors for the three species that were used in this thesis, analyses on biomass harvested at different seasons needs to be done. Marinho and Holdt [267] showed that the nitrogen-to-protein conversion factor for Danish *S. latissima* varied from 0.96-4.57 over a season. For Scottish *L. digitata*, *Laminaria hyperborea*, *S. latissima* and *Alaria esculenta*, the nitrogen-to-protein conversion factors varied between 3.1-6.6, 3.8-5.9, 2.9-7.1 and 5.7-6.2, respectively, over the season [69] and for Faroese *S. latissima* between 1.2-2.7 [86]. For pH-shift extracts from *P. umbilicalis* and *S. latissima*, the nitrogen-to-protein conversion factors were instead 6.2 and 7.7. These results indicate that non-protein-bound nitrogen was removed during the pH-shift process and therefore a higher conversion factor is needed for this type of seaweed extracts compared to dry seaweeds.

**Table 11:** Total amino acids, total protein content and nitrogen content (% dw) in seaweed and protein extracts in study III. Data are shown as mean values  $\pm$  stdv, (n=3), if not other is stated. x denotes that no analyse was performed.

	Total amino acids	Total Protein Lowry	Total nitrogen LECO	Total Protein LECO N/P factor: 5	Total nitrogen Kjeldahl	Total Protein Kjeldahl N/P factor: 5	Free nitrogen Kjeldahl <sup>#</sup>
<i>P. umbilicalis</i> dry	31.8 $\pm$ 1.7	31.4 $\pm$ 1.9	6.0 $\pm$ 0.01	30.2 $\pm$ 0.05	x	x	0.003 <sup>§</sup>
<i>P. umbilicalis</i> traditional	13.9 $\pm$ 0.2	18.1 $\pm$ 3.0	17.4	87.2	15.8 $\pm$ 0.1 <sup>*</sup>	78.9 $\pm$ 0.3 <sup>*</sup>	11.7 <sup>§</sup>
<i>P. umbilicalis</i> pH-shift	71.0 $\pm$ 3.7	97.6 $\pm$ 6.5	11.4 $\pm$ 0.7	57.1 $\pm$ 3.7	8.7 $\pm$ 0.1 <sup>*</sup>	43.3 $\pm$ 0.4 <sup>*</sup>	0.001 <sup>§</sup>
<i>P. umbilicalis</i> ASE	21.2 $\pm$ 0.6 <sup>*</sup>	42.2 $\pm$ 11.7	x	x	x	x	x
<i>U. lactuca</i> dry	19.6 $\pm$ 0.6	20.5 $\pm$ 0.1	3.7 $\pm$ 0.0	18.5 $\pm$ 0.00	x	x	0
<i>U. lactuca</i> traditional	10.7 $\pm$ 1.4	23.8 $\pm$ 2.5	14.7	73.6	14.2 $\pm$ 0.0 <sup>*</sup>	70.8 $\pm$ 0.1 <sup>*</sup>	10.5 $\pm$ 0.4 <sup>*</sup>
<i>U. lactuca</i> pH-shift	51.2 $\pm$ 2.1	87.3 $\pm$ 7.9	x	x	x	x	x
<i>U. lactuca</i> ASE	13.0 <sup>§</sup>	11.2 $\pm$ 1.8	x	x	x	x	x
<i>S. latissima</i> dry	10.1 $\pm$ 0.2	16.5 $\pm$ 0.0	2.0 $\pm$ 0.2	10.0 $\pm$ 0.9	x	x	0.001 <sup>§</sup>
<i>S. latissima</i> traditional	1.9 $\pm$ 0.1	5.6 $\pm$ 0.4	18.9	94.5	18.1 $\pm$ 0.1 <sup>*</sup>	90.4 $\pm$ 0.5 <sup>*</sup>	16.4 $\pm$ 0.8 <sup>*</sup>
<i>S. latissima</i> pH-shift	40.7 $\pm$ 0.5	82.4 $\pm$ 8.1	5.3	26.7	5.9 $\pm$ 0.1 <sup>*</sup>	29.3 $\pm$ 0.4 <sup>*</sup>	x
<i>S. latissima</i> ASE	5.0 $\pm$ 0.0 <sup>*</sup>	17.5 $\pm$ 3.4	x	x	x	x	x

<sup>\*</sup>Analysis was performed in duplicates. Data are shown as mean values  $\pm$  (max-min)/2, (n=2).

<sup>§</sup> Analysis was performed on single samples

<sup>#</sup>Analysis was performed on supernatant after water extraction (vortexing) followed by centrifugation.

## 5.5 Influence of protein extraction method on achieved protein yield

Protein yield in achieved extracts from study III was calculated both based on amino acid analyses and Lowry measurements. The obtained value differed depending on the analysis method used, however, the internal order among methods was kept. For both *P. umbilicalis* and *S. latissima* the protein yield ranked the methods as: pH-shift > traditional > ASE. For *U. lactuca* the order instead was traditional > pH-shift > ASE. The achieved yields can be seen in **Table 12**, which also includes the protein yield obtained for *P. umbilicalis* in study IV, and the highest achieved protein yield for *U. lactuca* in study V.

**Table 12:** Protein yields achieved from *Porphyra umbilicalis*, *Ulva lactuca* and *Saccharina latissima* when subjected to protein extraction methods in studies III-V. Data are shown as mean values  $\pm$  (max-min)/2, (n=2).

Study and analyse method the yield is based on	Protein Yield			
	Study III Lowry	Study III Amino acids	Study IV Dumas	Study V Lowry
<i>P. umbilicalis</i> traditional	5.2 $\pm$ 0.2	4.4 $\pm$ 0.2		
<i>P. umbilicalis</i> pH-shift	22.6 $\pm$ 7.3	16.8 $\pm$ 5.4	15.0 $\pm$ 4.8	
<i>P. umbilicalis</i> ASE	3.7	1.9		
<i>U. lactuca</i> traditional	19.6 $\pm$ 0.8	10.9 $\pm$ 1.9		
<i>U. lactuca</i> pH-shift	6.4 $\pm$ 3.1	4.2 $\pm$ 2.0		29.0 $\pm$ 6.9
<i>U. lactuca</i> ASE	0.4	0.7		
<i>S. latissima</i> traditional	3.1 $\pm$ 1.0	2.8 $\pm$ 1.7		
<i>S. latissima</i> pH-shift	25.1 $\pm$ 0.9	22.1 $\pm$ 0.8		
<i>S. latissima</i> ASE	0.7	0.4		

### 5.5.1 The traditional method (study III)

Protein yield obtained in study III with the traditional method were for *P. umbilicalis* 5.2% and 4.4% with Lowry and total amount amino acids, respectively. For *U. lactuca* and *S. latissima* the corresponding values were 19.6% and 10.9%, as well as 3.1% and 2.8%, respectively. No previous studies have reported on recovery of proteins from *P. umbilicalis*, but data from other red seaweed species exist. Using *Hyponea charoides* and *Hyponea japonica*, Wong and Cheng [30] applied the traditional method, with the use of mercaptoethanol and a pH rise to 12 before ammonium sulphate precipitation and subsequent re-extraction of the residue five times, and obtained 45.4-46.3% protein yield. The reported protein yield for *U. lactuca* in the same study [30] was 36%. Hence higher yields than ours were obtained, which could be due to our use of only two re-extractions of the residue. As mentioned earlier, the use of alkaline extraction compared to only water (as was used in study III) has been shown to have a large effect on the protein solubility. For example, for *U. rigida* and *Ulva rounded*, it increased to 26.8% and 36.1%, compared to 9.7% and 14.0% when using water only [149]. Accordingly, Harnedy and Fitzgerald [154] found that the extractability of *P. palmata* proteins increased from ~5.1 to 6.7 g/100g seaweed when the concentration of NaOH increased from 0.08 to 0.12 M. This was also seen during our adaption of the pH-shift process, where the solubilities at pH 12 for *P. umbilicalis* and *U. lactuca* were 54% and 62% compared to 34% and 35% at their respective native pH. The addition of mercaptoethanol to break disulphide bonds between proteins has

earlier been found to facilitate the extractability further [268]. However, the use of mercaptoethanol will render the process non-food-grade and was therefore not an alternative to use in study III.

Using the same method as Wong and Cheung [30], Kandasamy et al. [159] and Suresh Kumar et al. [160] reported protein yields for *Enteromorpha* species (*E.compressa*, *E.linza* and *E.tubulose*) of 5.71-6.48% [159] and for *Kappaphycus alvarezii*, 7.81% [160], hence in the same range as we obtained in study III for *P. umbilicalis* and *S. latissima*, but lower than for *U. lactuca*. Reported protein yield when using ammonium sulphate for precipitation without addition of 2-mercaptoethanol was 6.5% using the brown seaweed *H. elongate* [157]. However, in the latter study, it is not stated if the yield is per weight or initial protein content, rendering comparisons between methods difficult.

### 5.5.2 The pH-shift method (studies III-V)

The pH-shift process gave the highest total protein yield for *P. umbilicalis* (22.6% and 16.8% with Lowry and total amount amino acids, respectively) and *S. latissima* (25.1% and 22.1% with Lowry and total amount amino acids, respectively) in study III. For *U. lactuca*, the yield was significantly lower (6.4% and 4.2% with Lowry and total amount of amino acids, respectively). Using a similar process as the pH-shift process, but without a freeze-thawing step, Angell et al. [169] obtained a protein yield of 12.3% from *Ulva ohnoi*, hence higher than our results for *U. lactuca* in study III. They initially stirred the biomass in de-ionised water for 16h compared to the 1h we used in study III. A longer osmotic shock has been shown to facilitate protein solubilisation for *U. lactuca* at its native pH [156], something which was not seen for *S.latissima* at pH 12 by Veide Vilg and Undeland [148]. Other reported protein yields from dry *S. latissima* using the pH-shift process without a freeze-thawing step [124, 148] vary between 7.2 and 16.0%, and with a freeze-thawing step [124], from 12.0 to 26.4%. Using wet *S. latissima* biomass, the corresponding range, when a freeze-thawing step was included, was 16.6-19.3% [124]. Our obtained protein yield for *S. latissima* with the pH-shift process is thus placed in the higher end of the reported values.

#### *Effect of the enzyme ulvan lyase on the protein yield for U. lactuca during the pH-shift process (study V)*

The hypothesis in study V was that the use of ulvan lyase would break the cell wall and thereby increase the solubility of the proteins, however, this hypothesis could not be confirmed. Instead, when adding the enzyme into the process, the protein solubility unexpectedly decreased while the precipitation increased. This result could be explained in three different ways. Firstly, the shorter sulphated ulvan fragments formed during hydrolysis would be soluble in the water phase. Those charged fragments could have a salting-out effect on the proteins and thereby decrease the solubility and increase the precipitation. Secondly, at the low pH used for precipitation, it is likely that some of the extracted proteins will be positively charged and hence attracted to the charged sulphated groups. The larger complexes formed could more easily sediment. Lastly, the enzyme could have released some of the large structural proteins incorporated into the cell wall of *U. lactuca* [269], which could then work as ‘seeds’ for the smaller proteins stimulating co-precipitation. A similar phenomenon has been suggested for fish proteins during the pH-shift process, where myofibrillar proteins are suggested to act as seeds for the sarcoplasmic proteins and hence facilitate the precipitation [270].

Fleurence et al. [149] report decreased protein solubility when using cellulase to enhance the extractability of *U. rigida* and *U. rounded* proteins. By adding cellulase, less than 1% of the proteins was extracted, compared to 26.8 and 14.0% when extracting in water alone. In their study, only protein extraction yield was reported (i.e. total protein extracted/total proteins)\*100, as no precipitation was done. Their reported values are therefore comparable to the yield we obtained in the first step of the pH-shift process at RT, i.e. 32.9% and 54.6% with and without ulvan lyase, respectively. When they [149] instead used a polysaccharidase mixture (cellulase, hemicellulose and  $\beta$ -glucanase), 18.48% and 22% of the proteins were extracted from *U. rigida* and *U. rounded*, respectively. Fleurence et al. [149] argued that, to access the cellulose, cellulase might need an initial partial destruction of the cell wall and therefore could give a positive effect on protein extractability in combination with other enzymes. However, in a more recently published study [156], cellulase and pectinase added separately had a positive effect on protein extraction from *U. lactuca*, increasing it to ~25-30% compared to ~10-17% without enzymes. However, a mixture of the same enzymes had no effect compared to the control without enzymes. Despite this, since cellulase, alone or in combination with other enzymes, has shown a positive effect on the extractability of seaweed proteins, and since cellulose is the other main polysaccharide found in the cell wall of *Ulva* sp. [269], this enzyme in combination with ulvan lyase could be an alternative for further investigation.

#### *Effect of process temperature on the protein yield for U. lactuca*

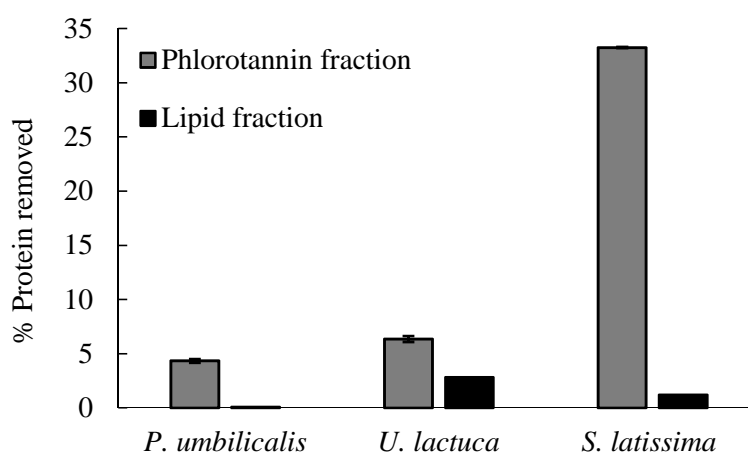
The temperature during the pH-shift process did not affect the protein solubility, but decreased temperature increased the precipitation and therefore also the total protein yield, see Figure 2 in study V. A tentative reason is that the protein pellet became firmer, and was easier to recover. No previous study has to the best of our knowledge reported on the effect of temperature on seaweed protein precipitation. Postma et al. [156] however found that an increase in temperature from either 4 or 22 °C to 30 °C during the osmotic shock step increased the extractability of *U. lactuca* proteins, regardless of the length of the osmotic shock (1, 4 or 24 h), however, the difference was clearer during the longer osmotic shock (24h). The total pH-shift process time elapsing until the first centrifugation step was only 80 min in study V, which could explain why solubility was not affected by temperature in this case.

#### *Effect of an additional incubation at pH 8.5 before further solubilisation at pH 12 on the protein yield for U. lactuca*

The extra incubation step at pH 8.5 included in study V, to activate the ulvan lyase, was found to increase both the protein solubility and precipitation significantly. It even showed better effect on the protein solubility than the prolonged incubation at pH 12 (80 min). In study III, we showed that the solubilisation of *U. lactuca* proteins starts immediately when the pH is raised from the native one (pH 4.8), see Figure 2 in study III, something which was confirmed here. Possibly, the proteins solubilising at pH 8.5 aided the precipitation of other proteins and hence also increases the precipitation. In combination with the lowest process temperature (8 °C), the extra incubation step at pH 8.5 for 60 min before further solubilisation at pH 12 for 20 min provided a final protein yield for *U. lactuca* of 29.0% in study V compared to 6.4% in study III (where this extra step was lacking).

### 5.5.3 The ASE method

The protein yield achieved using the novel ASE technique was low for all species (0.4-3.7% and 0.4-1.9% with Lowry and total amount amino acids, respectively), see **Table 12**. To verify the hypothesis presented in study III, that the proteins from *S. latissima* likely reacted with phlorotannins and thus were removed in the phlorotannin extraction step, the protein content of the phlorotannin and lipid extract emerging during the ASE process were analysed. The relative amount of protein removed in those steps is presented in **Figure 18**. A large portion of the proteins (33%) was removed in the phlorotannin extraction step for *S. latissima*, compared to for *P. umbilicalis* (4%) and *U. lactuca* (6%). This verifies the hypothesis and thus reveals that the ASE technique was not suitable for protein extraction.



**Figure 18:** Percent proteins removed in the phlorotannin and the lipid extraction step during the ASE process of *Porphyra umbilicalis*, *Ulva lactuca* and *Saccharina latissima*. Data are shown as mean values  $\pm$  (max-min)/2, (n=2), for the phlorotannin fraction. Analyses of lipid fractions were performed on single samples. Note that the lipid fraction of *P. umbilicalis* was also analysed, however, the low obtained value (0.07%) is poorly visible in the graph.

## 5.6 Differences in protein yield from seaweeds compared to crops and legumes

Our data for protein yields from seaweed in studies III-V, along with other reported seaweed protein yields, are generally low compared to reported values from protein extraction of legumes, such as soy [271]. In legumes, proteins are found in storage entities, hence making extraction easier. In comparison, protein storage is low in seaweed. Seaweeds are photosynthetic organisms and therefore carry a large quantity of their proteins inside the thylakoid membrane in highly interconnected structures.

The type of proteins differs between seaweed and legumes. Storage proteins mainly consist of four types of proteins [272]. For example, in soy and pea, 40-80% of the storage proteins is globulin, which is salt-soluble. The uniformity of the storage proteins makes the extraction easier. In photosynthetic tissue, the main classes of proteins are instead structural and enzymatic proteins. The enzymatic proteins are mainly found inside the thylakoids, connected to chlorophyll and carotenoids. Within this pool of proteins, there is a large heterogeneity due to their different roles in the photosynthesis. Therefore, it is not possible to use one or two extraction steps as for legumes and crops to extract the majority of the proteins from a photosynthetic matrix.

Also, as mentioned earlier, the polysaccharide-rich cell walls and the abundance of phenolic compounds in seaweed reduce the protein extractability [30, 150, 151]. Polyphenols can oxidise amino groups of peptides, leading to cross-links and the formation of reactive quinines. Quinines can react further with proteins, leading to the formation of large complexes [152]. Also, the phlorotannins found in brown seaweed reduce protein extractability by forming covalent bonds to them [153].

## 5.7 Composition differences between protein extracts and dry biomasses (study III)

To allow for a relatively simple extraction method which can be up-scaled, our goal has been to produce semi-pure multi-functional seaweed protein extracts with interesting nutritional and techno-functional properties, rather than very pure protein isolates. The latter would call for numerous purification steps and would create a very costly and complex process. The same approach has also been suggested by Tamayo Tenorio et al. [271]. The co-extraction of e.g. LC n-3 PUFAs, minerals and vitamins could enhance the value of the extracts. Therefore, besides the total protein and amino acid composition of the achieved extracts in study III, also their fatty acid composition and ash content were analysed.

### 5.7.1 Protein content

The obtained protein content (based on total amino acids) in the achieved extracts in study III varied from 1.9-71% depending on the species and protein extraction method applied, **Table 11** (section 5.4). Only the pH-shift process could concentrate the proteins in the produced extracts, and the concentration factors were 2.2, 2.6 and 4.0 for *P. umbilicalis*, *U. lactuca* and *S. latissima*, respectively. Few previous studies on protein extraction from seaweed have also precipitated the proteins after the extraction step and therefore there is limited data available on protein content in seaweed extracts to make a comparison with.

The total amino acid content in the extracts produced with the traditional method was 13.9%, 19.6% and 1.9% (dw basis) for *P. umbilicalis*, *U. lactuca* and *S. latissima*, respectively. In

previous studies using alkaline extraction with the addition of 2-mercaptoethanol, followed by ammonium sulphate precipitation, the achieved protein content in obtained extracts varied between 33-85% for *Enteromorpha* spp. [159], *Sargassum* spp. [122], *K. alvarezii* [160], *H. charoides*, *H. japonica* and *U. lactuca* [30]. These numbers were obtained from analysis of total nitrogen using a conversion factor of 6.25. Using the same protocol for traditional extraction of proteins as we used in study III, Garcia et al. [157] obtained *H. elongata* extracts with 63% protein based on total nitrogen analysis and 5.4% protein based on total amino acid analysis. This big difference between total nitrogen and amino acid data indicates that the dialyse step at the end of the traditional process was insufficient in removing the ammonium sulphate from the extracts, equal to what we found for the traditional extracts in study III (see section 5.4).

The total amino acid content in the extracts produced with the pH-shift method was 71.0%, 51.2% and 40.5% (dw basis) for *P. umbilicalis*, *U. lactuca* and *S. latissima*, respectively. Using a process similar to our pH-shift process, but without freeze-thawing, an extract from *U. ohnoi* was reported to contain 56% protein based on total amino acid analysis [169], hence slightly higher than the content we obtained for the *U. lactuca*. It was recently reported that the pH-shift process with a freeze-thawing step included rendered *S. latissima* extracts containing 15-38% protein depending on the type of pretreatment applied prior to processing (freezing at different temperatures and different drying techniques) [124]. The highest protein content (38%) was achieved in the freeze-dried biomass, which is similar to the content we obtained for *S. latissima*. To our knowledge, there are no earlier reports on extracted proteins from *P. umbilicalis*, but for the red *H. charoides* and *H. japonica*, extracts with 83% and 85% protein, respectively, were reported using alkaline extraction with the addition of 2-mercaptoethanol, followed by ammonium sulphate precipitation [30]. Based both on our results in study III and the available data from others, it is thus clear that alkaline extraction is superior to water extraction and enzyme assisted extraction [146] for delivering extracts high in proteins.

The ASE method produced extracts contained 21.2%, 13.0% and 5.0% total amino acids using *P. umbilicalis*, *U. lactuca* and *S. latissima*, respectively, hence lower than in respective crude seaweed biomass, **Table 11**. Based on the relatively low content of proteins and the low protein yields (0.4-1.9% based on analysis of total amino acids), it is concluded that the ASE method, when performed according to study III, is not suitable for producing protein extracts from those seaweeds.

### 5.7.2 Amino acid composition

For the traditionally made and the pH-shift-produced extracts, the relative amount of essential amino acids (36.8-40.7%) was slightly higher than in the respective crude seaweed biomass (34.7-38.1%). The amino acid pattern of the mentioned extracts was quite similar to the crude biomasses. The ASE extracts differed in composition from the other extracts primarily in two ways. Firstly, the relative amount of essential amino acids was lower (7.0-22.3%) and secondly, glutamic acid had been highly concentrated, 2.6, 2.4 and 1.9 times for *P. umbilicalis*, *U. lactuca* and *S. latissima*, respectively. Glutamic acid is the amino acid giving raise to the typical umami taste of seaweed [55, 56] and the high levels of glutamic acid in the ASE extracts thus open up for use as seasoning with potentially high umami taste.



### Amino acid chemical score

The achieved amino acid chemical score for the dry seaweeds used in study III, 22-89, **Table 13**, is lower or equal to numbers earlier reported for seaweed (51-106) [86, 273]. Since tryptophan was not detected with the used amino acid analysis method, this amino acid was not included in the calculations. For the pH-shift extracts, the scores were notably higher, 97-108, compared to the crude biomasses. This corresponds well with that the essential amino acids were concentrated during the pH-shift process and strengthens the hypothesis that seaweed is a promising source for new vegetarian proteins. For the traditional extracts, the amino acid chemical score increased for *P. umbilicalis* and *U. lactuca*, despite the fact that the total amino acid content in the extracts was lower than in corresponding seaweed biomasses. For all ASE extracts, the scores were lower, in accordance with their lower levels of essential amino acids compared to the crude seaweeds.

**Table 13:** Amino acid chemical score for *Porphyra umbilicalis*, *Ulva lactuca* and *Saccharina latissima* biomasses and protein extracts thereof in study III.

	Amino acid chemical score	Limited amino acid/s
<i>P. umbilicalis</i> dry	33	Histidine
<i>P. umbilicalis</i> traditional	91	Histidine
<i>P. umbilicalis</i> pH-shift	117	Lysine
<i>P. umbilicalis</i> ASE	5	Leucine
<i>U. lactuca</i> dry	22	Histidine
<i>U. lactuca</i> traditional	77	Lysine
<i>U. lactuca</i> pH-shift	97	Lysine
<i>U. lactuca</i> ASE	13	Lysine
<i>S. latissima</i> dry	89	Lysine
<i>S. latissima</i> traditional	40	Histidine
<i>S. latissima</i> pH-shift	108	Lysine
<i>S. latissima</i> ASE	38	Leucine

### 5.7.3 Fatty acid composition

For the pH-shift extracts of *U. lactuca* and *S. latissima* the total fatty acid content was significantly higher than in the crude seaweeds. For *U. lactuca*, it went from 2.3% dw in crude biomass to 5.0% dw in the pH-shift extract and for *S. latissima* it went from 2.1% in crude biomass to 3.3% in the pH-shift extract. For *U. lactuca*, the low n-6/n-3 ratio (0.27) found in the biomass was kept in the pH-shift extracts, **Table 7** (section 5.1.2). The relative amount of ALA was lower in the *U. lactuca* pH-shift extract (19.6%) compared to the crude biomass (25.0%), but instead, C16:4n3 was concentrated. For *S. latissima* the n-3 fatty acids exerted only 16.3% of total fatty acids in the pH-shift extract compared to 47.1% in the crude seaweed, resulting in a n-6/n-3 ratio of 1.18 for the pH-shift extracts compared to 0.48 for the crude seaweed. There was no significant difference in total fatty acids between crude *P. umbilicalis* and the pH-shift extract, and these samples also had similar n-6/n-3 ratios (0.58 respective 0.54). The co-extraction of fatty acids, especially of the n-3 family, could give an added value to the protein extracts. However, it is well known that PUFAs are susceptible towards oxidation, which could cause both an unpleasant off-flavour and the formation of unwanted oxidation

products [126, 129]. Therefore, the stability of the fatty acids, both during the actual process and during subsequent storage of the extracts, needs to be investigated. The traditionally made seaweed extracts and the ASE extracts had significantly lower fatty acid content than the respective crude seaweeds. In the case of the ASE method, it comprises an initial de-fatting step which probably explains the lower fatty acid content in those extracts

#### **5.7.4 Ash content**

The ash content of both the traditional extracts and the pH-shift extracts was significantly lower than in the crude seaweeds. This is probably due to the relatively large water volumes used during these processes, which dilute the minerals. The ash content in the ASE extracts was significantly higher than in respective crude seaweeds. In this process, there is no dilution since the extraction is made with acetone and water followed by evaporation. The ASE extracts could therefore rather be interesting from a mineral and salt-replacer point of view, contributing with a high saltiness and umami taste due to the high levels of minerals and glutamic acid (section 5.7.2).

### **5.8 Qualitative characterisation of polysaccharide fractions from the sequential recovery of macromolecules in study IV**

It was successfully proven in study IV that a biorefinery approach for recovery of several valuable compounds from *P. umbilicalis* is possible. A biorefinery concept would facilitate better use of the raw material compared to extractions targeting only single compounds. It also minimises transportation of residues, since all extractions would be done in the same location, meanwhile contributing to the concept of circular economy [274, 275]. Depending on the specific seaweed species, a wide range of high-value products could be produced. In study IV, we applied sequential extraction of proteins, carrageenan, pectin and cellulose from *P. umbilicalis* and thus demonstrated an example of a biorefinery approach. Baghel et al. [276] recovered phycobilin pigments, lipids, agar, and a mineral-rich liquid from *Gracilaria corticate* using cold aqueous extraction, organic solvent extraction and hot aqueous extraction. Bioethanol was produced from the residual after the extractions using saccharification and fermentation. The authors [276] also concluded that the final residue could be used as a soil conditioner. Further, Glasson et al. [277] extracted salt, pigments and ulvan from *U. ohnoi* using aqueous extraction, ethanol extraction and acidic extraction. These examples illustrate the variety of valuable compounds that can be extracted from seaweed biomass using a biorefinery concept. An important aspect to consider in such biorefining is, however, to recover sensitive molecules first, so that e.g. harsh solvents or high temperatures do not cause degradation or, in the case of proteins, denaturation. In study IV, we applied the pH-shift process first for this reason and then subjected the non-soluble fraction to hot alkaline, acidic and organic solvent extractions, all of which could denature or cause other non-favourable reactions to the proteins.

The carrageenan-rich fraction extracted in study IV formed a gel with  $K^+$  but not  $Ca^{2+}$ , Figure 7 in study IV, indicating that the major form was  $\kappa$ -carrageenan. The ability of the carrageenan fraction to form a gel makes it interesting to investigate further, for future use as a thickening agent in e.g. food and/or in hydrogel applications. Food-grade carrageenan is today produced industrially from other red species (*Kappaphycus* spp. and *Eucheuma* spp.) [43, 103] and therefore it is not likely that *P. umbilicalis* would be used for this purpose only. However, in a biorefinery approach where carrageenan is one out of several targeted compounds, it could add value to the *Porphyra umbilicalis* biomass.

A pectin fraction was unexpectedly recovered from *P. umbilicalis*. No one has to our knowledge previously reported on pectin extraction from seaweed, although it has earlier been reported in the cell wall of *Ulva compressa* [278]. Pectin, normally produced from citrus [279], is used within the food industry as a thickening agent due to its ability to form gels together with  $\text{Ca}^{2+}$ . The obtained pectin fraction in study IV had a low galacturonic acid content compared to the pectin obtained from citrus peel, and hence, the gelling capacity of this fraction could be different, which is still to be explored.

A small cellulose fraction was recovered at the end of the sequential extraction of proteins, carrageenan and pectin; it contributed to 1.2% of the initial weight of the biomass. Due to the very low amount, *P. umbilicalis* is not a promising source for cellulose production. Other seaweed species such as *Gelidiella acerosa* and *Sargassum tenerrimum* might be more suitable for this purpose [107].

## 6. CONCLUSIONS

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This thesis evaluated seaweeds as a potential food protein source. The results strengthen the hypothesis that seaweed could be an important contributor to the protein shift, meanwhile also contributing with PUFAs, vitamins and minerals. More precisely, the results showed that:

- The protein and fatty acid content in *U. lactuca* biomass were increased by low light and the addition of nitrate during cultivation. Lower temperature (13 °C) increased the protein content, while high temperature (18 °C) increased the fatty acid content. These results show that by alternative cultivation, protein-enriched *U. lactuca* could be obtained for further use e.g. in protein extractions.
- There was a moderate development of the aldehydes MDA, HHE and HNE in *U. lactuca* and *P. umbilicalis* up to 520 days of storage. Along with this, there was a drastic loss of fatty acids. Further, ascorbic acid decreased in all samples during a storage period of 230 days and it was found that the whey coating significantly reduced the levels of ascorbic acid in both *P. umbilicalis* and *U. lactuca* during the actual drying step. There were also significant losses of green and yellow colour during storage of the dried seaweeds. Light stimulated the development of aldehydes as well as the loss of ascorbic acid (in *U. lactuca*), fatty acids and colour. Thus, dried seaweed should be stored in darkness to keep nutrients intact and the storage period should be as short as possible.
- The three seaweed species responded differently to the three protein extraction methods evaluated. The pH-shift method gave the highest total protein yield for *P. umbilicalis* (23%) and *S. latissima* (25%), while the traditional method gave the highest total protein yield for *U. lactuca* (20%). The ASE method resulted in very low protein yields for all three species and it is concluded that this method is not suitable for protein extraction from these seaweed species.
- The protein yield during pH-shift processing of *U. lactuca* could be increased from 9.2% to 29%, hence higher than achieved with the traditional method, by an extra incubation step at pH 8.5 and 8 °C, before further protein solubilisation at pH 12. Surprisingly, the addition of the enzyme ulvan lyase during pH-shift processing of *U. lactuca* significantly decreased the protein solubility but increased the precipitation. The net effect was that the total protein yield was not raised beyond the yield provided by the extra incubation step alone.
- All pH-shift extracts contained significantly more proteins (up to 71% dw) than the crude seaweed biomasses. The *U. lactuca* and *S. latissima* pH-shift extracts also contained significantly higher amounts of fatty acids than the crude seaweed biomasses. The traditional method did not concentrate proteins, fatty acids or ash for any of the used seaweed species. The ASE method concentrated the ash for all three species, but not proteins and lipids. Based on these results, the pH-shift method is identified to be a promising strategy for concentrating proteins from *P. umbilicalis*, *U. lactuca* and *S. latissima*.

- The protein analysis methods used: total amino acids, Lowry or nitrogen analysis using a nitrogen-to-protein conversion factor of 5, had little effect on the obtained results for crude *P. umbilicalis* and *U. lactuca*. However, for *S. latissima* the Lowry method resulted in higher values compared to the other methods, probably due to the abundance of phlorotannins. For the protein extracts the achieved protein content varied excessively with protein analysis method. Those results emphasise the importance of carefully selecting a protein analysis method to apply on seaweed and seaweed protein extracts.
- The pH-shift method could successfully be used as the first step in a sequential recovery of protein, carrageenan, pectin and cellulose from *P. umbilicalis*. The extracted carrageenan-fraction could be useful e.g. in hydrogel applications due to its gel-forming capacity together with  $K^+$ . These results pinpoint the possibilities to fully use the seaweed biomass for multiple products, without creating side streams.

## 7. FUTURE PERSPECTIVES

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The results presented in this work showed that the pH-shift process produced protein extracts high in proteins from seaweeds. The major incentive for extracting the proteins was that the seaweed protein digestibility is described to be low due to the polysaccharide-rich cell wall and the abundance of polyphenols in seaweed [23-30]. By extracting the proteins, we hypothesised that the digestibility could be increased. To test this hypothesis, a follow-up study should be performed where the digestibility of our produced seaweed extracts is to be compared to the digestibility of crude seaweeds. This is similar to what earlier has been done for pH-shift extracts from microalgae [280]. Furthermore, the protein bioavailability of extracts and crude seaweed should be investigated, for instance, using a caco-2 cell model.

To further raise the potential of the pH-shift process, additional experiments to increase the protein yield, both by increasing the solubility and the precipitation, should be carried out. For *U. lactuca*, it has previously been shown that an extended osmotic shock could increase the total yield [156], something that was not seen for *S. latissima* [148]. Based on this result, it could be interesting to further evaluate the combination of an extended osmotic shock and the pH-shift process for both *U. lactuca* and *P. umbilicalis*. In several studies [144, 151, 156] it has been shown that cellulase alone, or enzyme mixtures containing cellulase, have increased the extractability of seaweed proteins. This should be evaluated further by combining cellulase with ulvan lyase for *U. lactuca*, to increase the yield further. The use of polyphenol oxidase inhibitors could be an alternative to increase the protein yield by limiting interactions between proteins and polyphenols. To increase the protein precipitation, the use of flocculants at the end of the process is an interesting approach to evaluate.

We hypothesise that the observed concentration of lipids together with the proteins for *U. lactuca* and *S. latissima* in the pH-shift process is an extra added value to the extracts. Due to the unsaturated character of the lipids, their stability, during processing, subsequent handling and storage of the extracts must, however, be ensured. This is a point of further investigation. The value of the extracts could also be raised by the occurrence of vitamins and specific bioactive compounds, such as peptides and therefore a more thorough characterisation of the extracts is warranted. Also, due to the relatively low protein yield in the pH-shift process, it is very important to look into the side fractions of the process and evaluate also their potential application as high-value food or feed ingredients. This would secure economic revenue from investing in costly process equipment.

The techno-functionality, such as gelling capacity, water- and oil-holding capacity of the protein extracts is still to be explored. These properties are extremely important for the possible application areas of the protein extracts. Furthermore, sensory evaluation of the achieved extracts should be carried out to explore their suitability as potential food ingredients.

High iodine levels have been reported from brown seaweed species [115, 116]. Iodine is essential for us but could in high doses be toxic and therefore careful mapping of the partitioning of iodine during the pH-shift process is needed. Also, the ability of the seaweeds to take up heavy metals from the surrounding waters requires a very careful control of their partitioning in the pH-shift process. This is especially important in a potential future commercialisation.

Highest protein content was achieved in the biomasses cultivated during winter time. Also, addition of nutrients and low light and low temperature had a beneficial effect on the protein content during alternative cultivation of *U. lactuca*. More experiments where temperature and light are thoroughly explored together with the addition of nutrients could give a better understanding of the relationship between those parameters for the intracellular composition of *U. lactuca*.

In this work, only a moderate development of MDA, HHE and HNE was detected during storage of *P. umbilicalis* and *U. lactuca*, despite the substantial decrease in unsaturated fatty acid during storage, especially in light. A more expanded analysis of lipid oxidation products, such as e.g. hexanal, pentanal, nonanal, butanal and pentenol, could give additional answers regarding the stability of seaweed lipids during storage.  $\alpha$ -tocopherol is an efficient antioxidant by its ability to scavenge free radicals in synergy with ascorbic acid [281]. This quality makes it an interesting parameter to follow as an early oxidation marker during storage of seaweed.

For the carbohydrate fractions resulting from the sequential recovery of macromolecules from *P. umbilicalis*, no quantitative analysis was possible to perform. Because of this, only weight yields were reported for these fractions. Quantitative analysis methods would be required to further evaluate the yields of these polysaccharides, and hence make better conclusions on the biorefinery potential of *P. umbilicalis*.

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